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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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**INVENTOR(S)**

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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☐ Additional inventors are being named on the \_\_\_\_\_ separately numbered sheets attached hereto**TITLE OF THE INVENTION (280 characters max)****NEW METHODS OF DETECTING Lp-PLA2 ACTIVITY****CORRESPONDENCE ADDRESS**

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**ENCLOSED APPLICATION PARTS (check all that apply)**

- ☒ Specification Total Number of Pages  ☐ CD(s), Number
- ☒ Drawing(s) Number of Sheets  ☐ Other (specify)
- ☐ Application Data Sheet. See 37 CFR 1.76

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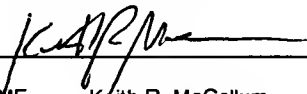
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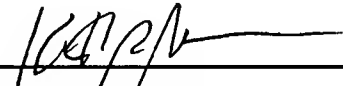
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- 2) Application consisting of 62 pages including; 42 pages of Specification including, 11 pages of Claims, and 1 page of Abstract; with 8 pages of Figures; and
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\_\_\_\_\_  
Keith R. McCollum  
Registration No. 53,294

## NEW METHODS OF DETECTING Lp-PLA2 ACTIVITY

## FIELD OF THE INVENTION

This invention relates to methods for determining the activity of Lipoprotein Associated Phospholipase A2 (Lp-PLA2). Specifically, it relates to determining the activity of Lp-PLA2  
5 by use of Lp-PLA2-specific binders and/or substrates capable of being converted into a detectable product in various formats. Furthermore, this invention relates to a hybrid-immunocapture activity assay for specifically determining the activity of Lp-PLA2.

## BACKGROUND OF THE INVENTION

## Introduction

10 Lipoprotein Associated Phospholipase A2 (Lp-PLA2) is an enzymatically active 50 kD protein. Lp-PLA2 is a member of the phospholipase A2 family, and unlike most phospholipases, is  $\text{Ca}^{2+}$  independent. Lp-PLA2 has been previously identified and characterized by Tew et al. (1996), Caslake et al. (2000), and in WO 95/00649-A1, US 5,981,252, US 5,968,818, US 6,177,257 (SmithKline Beecham) and WO 00/24910-A1, US  
15 5,532,152, US 5,605,801, US 5,641,669, US 5,656,431, US 5,698,403, US 5,977,308 (ICOS Corporation) which are herein incorporated by reference. Lp-PLA2 is expressed by macrophages, with increased expression in atherosclerotic lesions (Hakkinen 1999). Lp-PLA2 circulates bound mainly to LDL, co-purifies with LDL, and is responsible for >95% of the phospholipase activity associated with LDL (Caslake 2000).

20 In recent studies, lipoprotein-associated phospholipase A2 (Lp-PLA2) levels have been shown to be significantly correlated in men with angiographically-proven Coronary Heart Disease (CHD) (Caslake 2000) and associated with cardiac events in men with hypercholesterolemia (Packard 2000).

Coronary heart disease (CHD) is the single most prevalent fatal disease in the United  
25 States. In the year 2003, an estimated 1.1 million Americans are predicted to have a new or recurrent coronary attack (see the American Heart Association web site, [www.americanheart.org](http://www.americanheart.org)). Approximately 60% of these individuals have no previously known risk factors. It apparent there is a great need to diagnose individuals at risk of developing CHD and monitor response to therapies directed and reducing the individuals risk.

Previously, various methods for detecting Lp-PLA2 have been reported which include immunoassays (Caslake, 2000) and activity assays (PAF Acetylhydrolase Assay Kit, Cat#760901 product brochure, Cayman Chemical, Ann Arbor, MI, 12/18/97 ([www.caymanchem.com](http://www.caymanchem.com)) ; Kosaka, 2000). Additionally, the United States Food and Drug

5 Administration (FDA) has granted approval for an ELISA test for the quantitative determination of Lp-PLA2 in human plasma to be used as a predictor of risk for coronary heart disease (CHD) ((2003) Sep-Oct; New test predicts heart risk. FDA Consum. 37(5):6.).

Each of these assay formats has limitations. Assays which measure only enzymatic activity suffer from competitive activity for the substrate by other enzymes or substances  
10 present in the test sample. For instance, many members of the Phospholipase A2 family show enzymatic activity toward oxidized phosphatidylcholine. Additionally, the Cayman activity assay suffers from background signal due to substances in serum which metabolize the substrate independent of Lp-PLA2 activity. These assays can report erroneously high activity due to the lack of specificity. False measurements of activity in a clinical setting may lead to  
15 improper diagnosis of disease, or a patient's response to a therapy intended to reduce enzymatic activity.

In contrast, standard antibody based immunoassays are highly specific and capable of detecting and quantifying the amount of a target of interest amongst other closely related proteins. However, they are not capable of determining the level of enzymatic activity of the  
20 target. While this assay format ensures only the protein of interest is being measured, this limitation precludes such assays from being useful tools in monitoring a response to an enzyme inhibitor.

Accordingly, there is a great need for an assay capable of specifically selecting Lp-PLA2 from amongst other PLA2 family members which is further able to measure the  
25 enzymatic activity of Lp-PLA2.

### **Background Information on Coronary Heart Disease**

Coronary vascular disease (CVD) encompasses all diseases of the vasculature, including high blood pressure, CHD, stroke, congenital cardiovascular defects and congestive heart failure. Studies have shown that CHD is responsible for the majority of the CVD. The  
30 prevalence of CHD increases markedly as a function of age, with men having a higher prevalence than women within most age groups.

The current standard of care used to identify individuals at risk for heart disease is the measurement of a lipid panel, including triglycerides, total cholesterol, low density lipoprotein (LDL)-cholesterol, and high density lipoprotein (HDL)-cholesterol (Adult Treatment Panel III). According to the recent National Institutes of Health's, National Heart, Lung, and Blood Institute (NIH/NHLBI) publication; Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults, Adult Treatment Panel III (ATP III) guidelines (2001), depending on the risk factor score, individuals with LDL-cholesterol levels from  $\geq 100$  to  $\leq 130$  mg/dL are recommended to initiate therapeutic lifestyle changes. Adults with LDL-cholesterol  $> 130$  mg/dL are recommended for intensive lifestyle therapy and an LDL-cholesterol-lowering drug therapy to achieve an LDL-cholesterol goal of  $< 100$  mg/dL. Patients with LDL levels  $> 160$  mg/dL should be considered for therapies with lipid-lowering drugs. The American Heart Association has estimated that over 100 million adults in the US exceed the optimal level of total cholesterol ([www.americanheart.org](http://www.americanheart.org)).

While research continues to link elevated LDL-cholesterol levels with CHD risk, it is well understood that a significant number of individuals with normal LDL-cholesterol levels experience a cardiac event (Eaton 1998), suggesting that other factors not currently recognized may be involved. In the search for new risk factors, significant attention has been focused in recent years on markers of inflammation, as a growing body of basic and clinical research emerges regarding the role of inflammation in atherogenesis (Lusis 2000, Lindahl 2000). Some of the inflammatory markers under investigation include cell adhesion molecules, CD-40 ligand, interleukin 6 and C-reactive protein (CRP, measured by the high sensitivity method, or hsCRP). CRP, a non-specific acute phase inflammatory marker, has recently received significant attention as a potential risk indicator for CHD (Ridker 2002, Blake 2002). CRP, however, is well known to be responsive to many sources of inflammation, which justifies further investigations to identify more specific markers of arterial involvement.

The pathogenesis of atherosclerosis leading to the formation of unstable plaque has been recognized as one of the major causes of CHD (Lusis 2000). Recently, new understanding of the pathogenesis of atherosclerosis has placed emphasis on the inflammatory process as a key contributor to the formation of unstable plaque. The instability of the atherosclerotic plaque, rather than the degree of stenosis, is considered to be the primary culprit in the majority of myocardial infarctions (MI). This realization has led to the investigation of plaque biology and recognition that markers of inflammation may be useful as predictors of

cardiovascular risk. Among the various candidate markers of inflammation, CRP (measured by high sensitivity method, hs-CRP), a non-specific acute phase inflammatory marker, has received the most attention as a predictor of CHD (Ridker 2002).

### Scientific Review

5           Oxidation of LDL in the endothelial space of the artery is considered a critical step in the development of atherosclerosis. Oxidized LDL, unlike native LDL, has been shown to be associated with a host of pro-inflammatory and pro-atherogenic activities, which can ultimately lead to atherosclerotic plaque formation (Glass 2001, Witztum 1994). Increasing evidence from basic research suggests that atherosclerosis has an inflammatory component and represents  
10 much more than simple accumulation of lipids in the vessel wall. The earliest manifestation of a lesion is the fatty streak, largely composed of lipid-laden macrophages known as foam cells. The precursors of these cells are circulating monocytes. The ensuing inflammatory response can further stimulate migration and proliferation of smooth muscle cells and monocytes to the site of injury, to form an intermediate lesion. As layers of macrophages and smooth muscle  
15 cells accumulate, a fibrous plaque is formed, which is characterized by a necrotic core composed of cellular debris, lipids, cholesterol, calcium salts and a fibrous cap of smooth muscle, collagen and proteoglycans. Gradual growth of this advanced lesion may eventually project into the arterial lumen, impeding the flow of blood. Further progression of atherosclerosis may lead to plaque rupture and subsequent thrombus formation, resulting in  
20 acute coronary syndromes such as unstable angina, MI or sudden ischemic death (Davies 2000, Libby 1996).

          Lp-PLA2 plays a key role in the process of atherogenesis by hydrolyzing the sn-2 fatty acid of oxidatively modified LDL, resulting in the formation of lysophosphatidylcholine and oxidized free fatty acids (Macphee 1999). Both of these oxidized phospholipid products of Lp-  
25 PLA2 action are thought to contribute to the development and progression of atherosclerosis, by their ability to attract monocytes and contribute to foam cell formation, among other pro-inflammatory actions (Macphee 2001, Macphee 2002).

### Clinical Review

          Lp-PLA2 has been previously reported as a potential risk factor for CHD. The  
30 predictive value of plasma levels of Lp-PLA2 for CHD has been reported in a large, prospective case-control clinical trial involving 6,595 men with hypercholesterolemia, known

as the West of Scotland Coronary Prevention Study (WOSCOPS) (Packard 2000). Lp-PLA2 was measured in 580 CHD cases (defined by non-fatal MI, death from CHD, or a revascularization procedure) and 1,160 matched controls. The results indicated that plasma levels of Lp-PLA2 were significantly associated with development of CHD events by univariate and multivariate analyses, with almost a doubling of the relative risk for CHD events for the highest quintile of Lp-PLA2 compared to the lowest quintile. The association of Lp-PLA2 with CHD was independent of traditional risk factors such as LDL-cholesterol and other variables. This study provided an encouraging preliminary indication of the clinical utility of Lp-PLA2 as a risk factor for CHD.

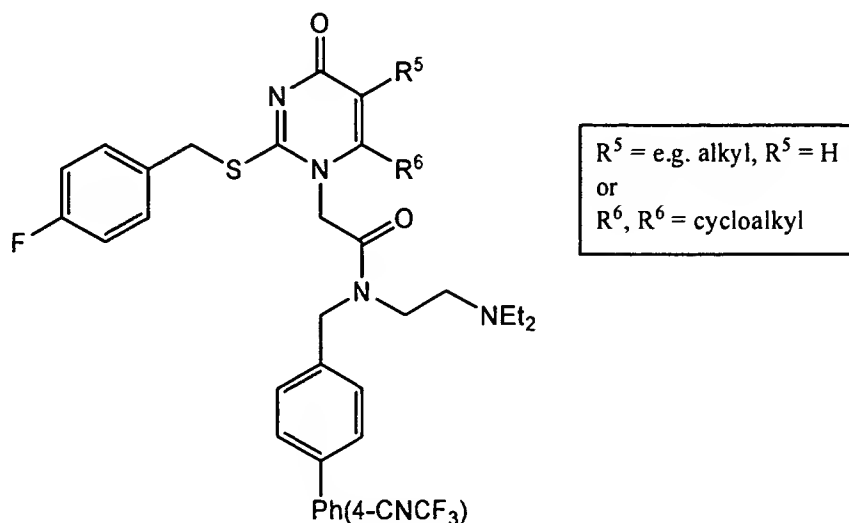
In a study of angiographically proven CHD, Lp-PLA2 was shown to be significantly associated with the extent of coronary stenosis (Caslake 2000).

In another study, in which only females were examined (n=246, 123 cases and 123 controls), baseline levels of Lp-PLA2 were higher among cases than controls (p=0.016), but was not significantly associated with CHD when adjusted for other cardiovascular risk factors.

In this study, cases included 40% of women with stroke, 51% non-fatal myocardial infarction and 9% fatal CHD (Blake 2001).

Furthermore, several papers have been published citing the potential of Lp-PLA2 as a therapeutic target for the treatment of coronary artery disease and atherosclerosis (Caslake 2000; Macphee 2001; Carpenter 2001; Leach 2001). Supporting the assertions that Lp-PLA2 is a therapeutic target for the treatment of CHD many articles have been published disclosing several genres of inhibitors of Lp-PLA2 and their use. These genres include but are not limited to: azetidinone inhibitors, SB-222657, SB-223777 (MacPhee 1999); reversible 2-(alkylthio)-pyrimidin-4-ones (Boyd, Flynn 2000); natural product derived inhibitors, SB-253514 and analogues (Pinto 2000); inhibitors produced by *Pseudomonas fluorescens* DSM 11579, SB-253514 and analogues (Thirkettle, Alvarez 2000; Busby 2000; Thirkettle 2000); 2-(alkylthio)-pyrimidones, orally active 1-((amidolinked)-alkyl)-pyrimidones (Boyd, Fell 2000); modified pyrimidone 5-substituent in 1-((amidolinked)-alkyl)-pyrimidones is highly water soluble (Boyd, Hammond 2001); Phenylpiperazineacetamide derivative of lipophilic 1-substituent in 1-((amidolinked)-alkyl)-pyrimidones (Bloomer 2001); 5-(Pyrazolylmethyl) derivative and 5-(methoxypyrimidinylmethyl) derivative of 1-(biphenylmethylamidoalkyl)-pyrimidones (Boyd, Fell 2002); Cyclopentyl fused derivative, SB-480848, of the pyrimidone 5-substituent in clinical candidate SB-435495 (Blackie 2003). To date, GlaxoSmithKline (GSK) has

announced positive clinical data for a novel compound, shown below, that dramatically lowers Lp-PLA2 activity. This Lp-PLA2 inhibitor may represent a new generation of drugs that reduce cardiovascular disease and death.



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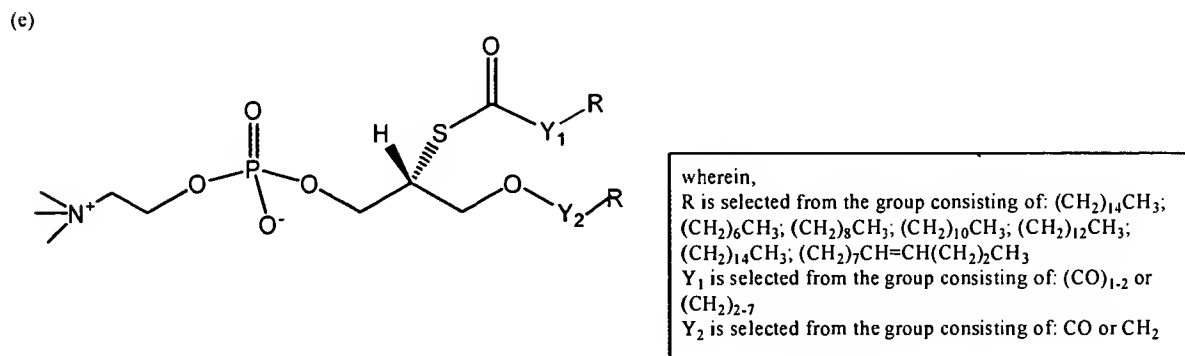
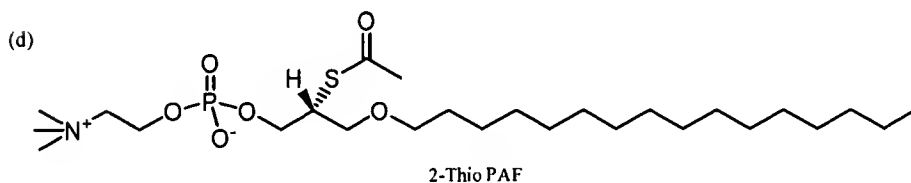
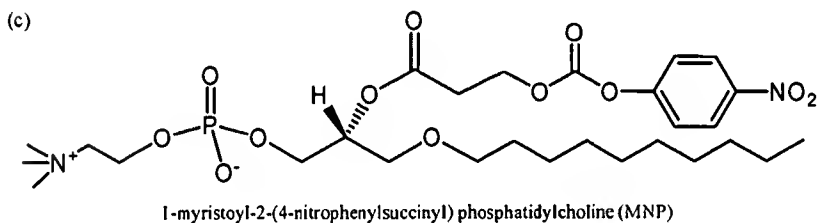
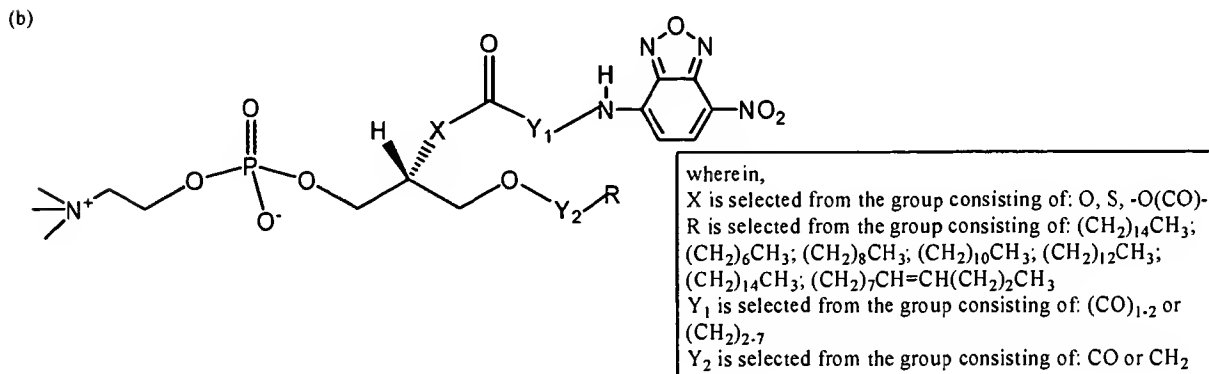
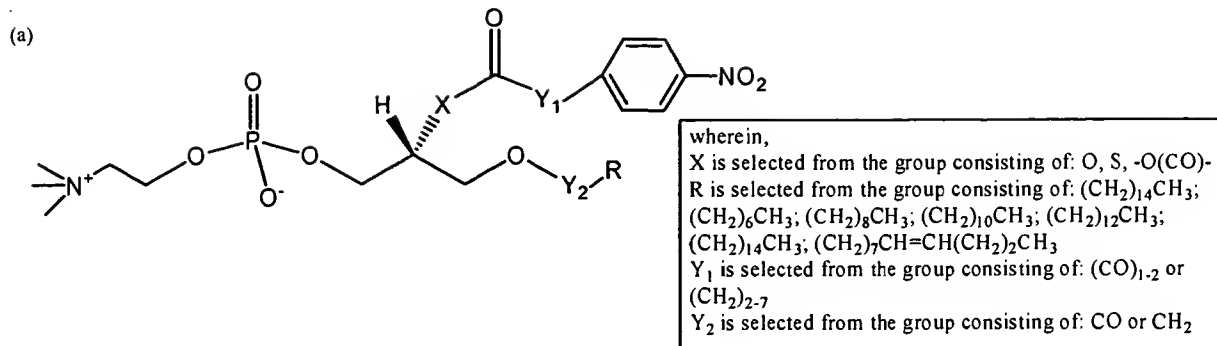
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### SUMMARY OF THE INVENTION

- 15 This method is directed to a method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising contacting an immobilized binder, which specifically binds Lp-PLA2, with the sample; washing the immobilized binder to remove an enzymatically active unbound material or an interfering substance(s); contacting the bound Lp-PLA2 with a substrate converted to a detectable product in the presence of Lp-PLA2; and
- 20 measuring detectable product indicative of enzymatically active Lp-PLA2 in the sample. In one aspect of the invention the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample. In another aspect of the invention the immobilized binder is an antibody. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred
- 25 aspect of the invention the monoclonal antibody is 2C10, 4B4, B200, B501, 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.

- In another aspect of the invention the enzymatically active unbound material is a phospholipase. In a further aspect of the invention the interfering substance(s) is a free-thiol compound. In yet another aspect of the invention the substrate is selected from the group
- 30 consisting of:



In a further aspect of the invention the substrate is an oxidized derivative of (a), (b), (c), (d) or (e) above.

In another aspect of the present invention the detectable product has a radioactive, colorimetric, paramagnetic or fluorescent label. Further, the detectable product is measured  
5 flourimetrically, colormetrically, paramagnetically or via radiation.

A further aspect of the invention comprises comparing the measured detectable product to detectable product in a control comprising an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the  
10 recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system. In another aspect of the present invention the immobilized binder is bound to a multi-well plate, a magnetic bead, or a latex bead.

This invention is also directed to a method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising: contacting an binder, which  
15 specifically binds Lp-PLA2, with the sample to form a binder-Lp-PLA2 complex; immobilizing the binder-Lp-PLA2 complex; washing the immobilized binder-Lp-PLA2 complex to remove an enzymatically active unbound material or an interfering substance(s); contacting the immobilized bound Lp-PLA2 with a substrate converted to a detectable product in the presence of Lp-PLA2; and measuring detectable product indicative of enzymatically  
20 active Lp-PLA2 in the sample. In one aspect of the invention the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample. In another aspect of the invention the binder is an antibody. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the monoclonal antibody is 2C10, 4B4, B200, B501,  
25 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.

In another aspect of the invention the binder-Lp-PLA2 complex is immobilized by binding to an immobilized compound. In a further aspect the immobilized compound is an antibody. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the  
30 monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody.

In another aspect of the invention the immobilized compound is bound to a multi-well plate, a magnetic bead, or a latex bead.

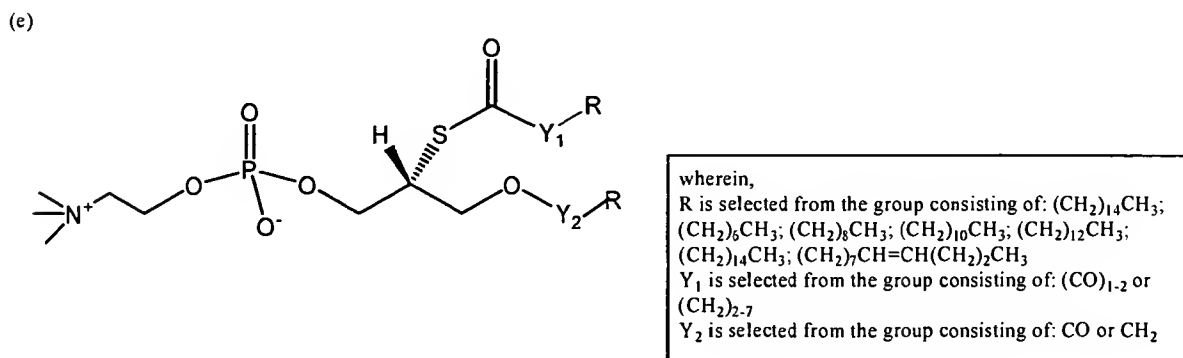
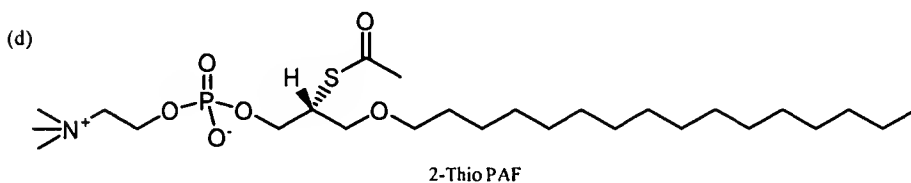
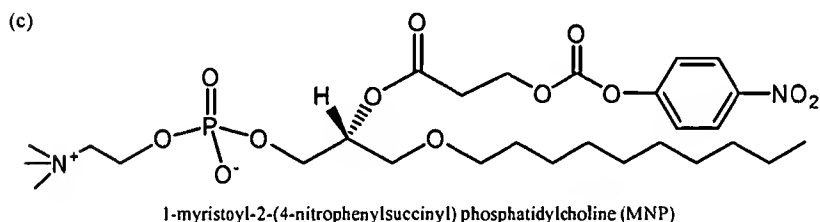
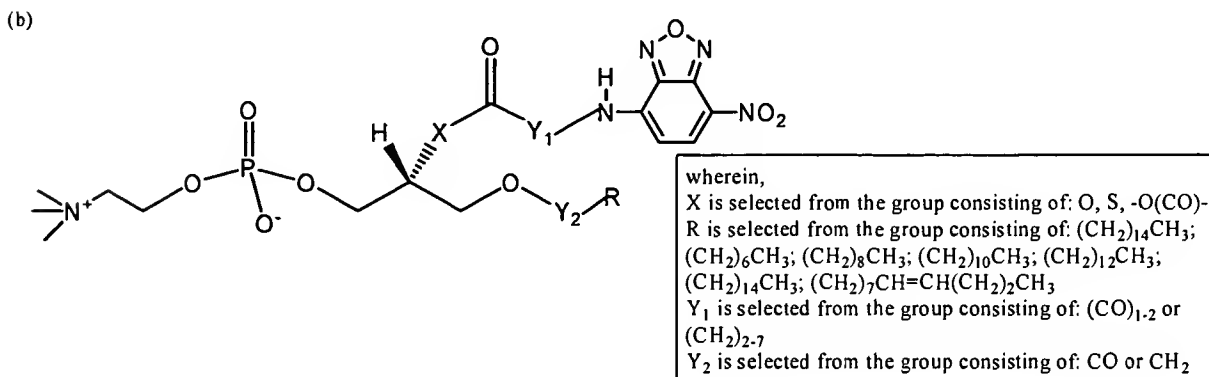
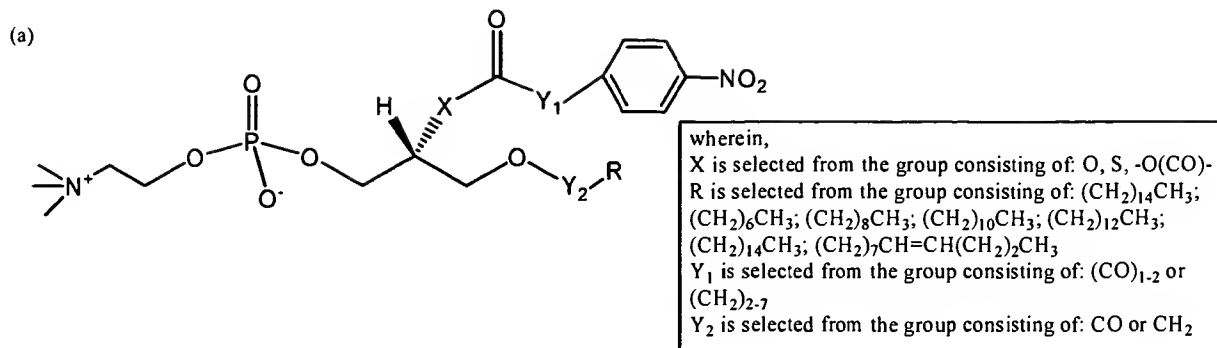
In another aspect of the invention the binder is conjugated to an immobilizing agent. In a further aspect of the invention the binder conjugated to an immobilizing agent is an antibody.

5 . In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the monoclonal antibody is 2C10, 4B4, B200, B501, 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.

10 In another aspect of the invention the immobilizing agent is an antibody, protein or compound capable of binding an immobilized compound. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody. In another highly preferred aspect the immobilizing agent is biotin.

15 In a further aspect of the invention the immobilizing agent, conjugated to the binder-Lp-PLA2 complex, binds to an immobilized compound. In a preferred aspect the immobilized compound is bound to a multi-well plate, a magnetic bead, or a latex bead. In another aspect of the invention the bound compound is an antibody, protein or compound capable of binding the conjugated immobilizing agent. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred  
20 aspect of the invention the monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody. . In another highly preferred aspect the immobilizing agent is streptavidin.

25 In another aspect of the invention the enzymatically active unbound material is a phospholipase. In another aspect of the invention the interfering substance(s) is a free-thiol compound. In yet another aspect of the invention the substrate is selected from the group consisting of:

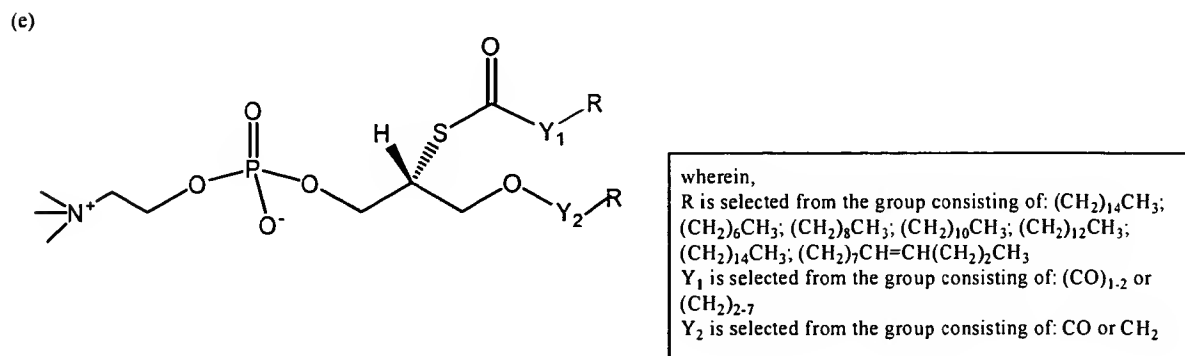
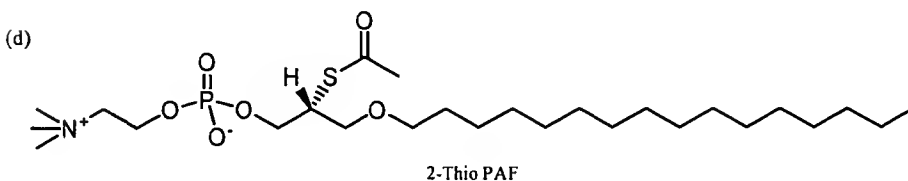
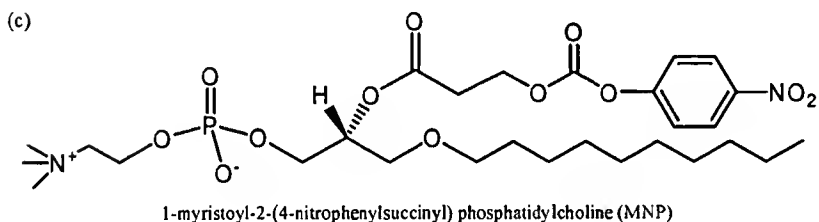
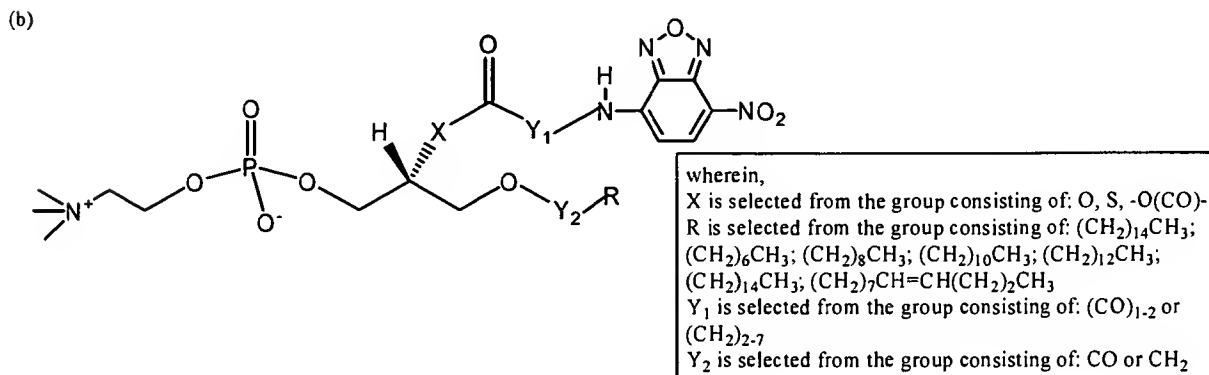
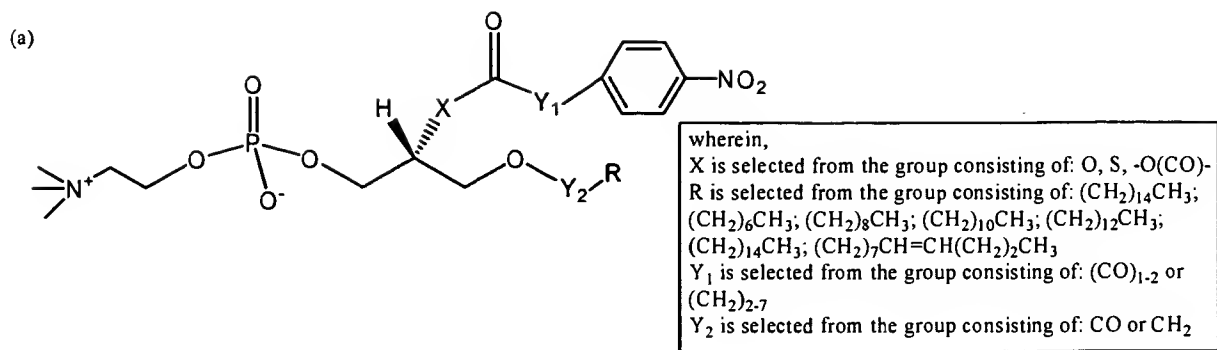


In a further aspect of the invention the substrate is an oxidized derivative of (a), (b), (c), (d) or (e) above.

In another aspect of the present invention the detectable product has a radioactive, colorimetric, paramagnetic or fluorescent label. Further, the detectable product is measured  
5 flourimetrically, colormetrically, paramagnetically or via radiation.

A further aspect of the invention comprises comparing the measured detectable product to detectable product in a control comprising an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the  
10 recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system. In another aspect of the present invention the immobilized binder is bound to a multi-well plate, a magnetic bead, or a latex bead.

The invention is also directed to a kit for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising a binder which specifically binds Lp-  
15 PLA2 and a substrate converted to a detectable product in the presence of Lp-PLA2. In another aspect of the invention the the substrate is selected from the group consisting of:



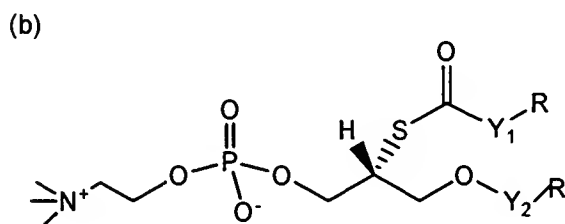
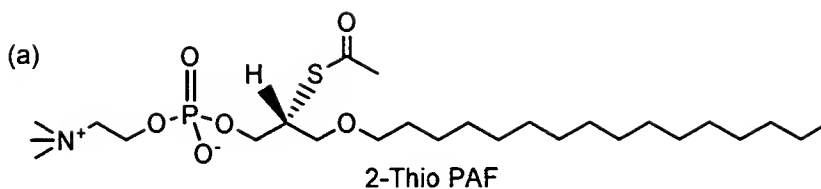
In a further aspect of the invention the substrate is an oxidized derivative of (a), (b), (c), (d) or (e) above. Another aspect of the invention is a kit comprising an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.

The invention is also directed to a method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising: incubating the sample with a compound which reduces active thiol(s) in the sample; contacting the incubated sample with a substrate converted to a free thiol product in the presence of enzymatically active Lp-PLA2; and measuring free thiol product indicative of enzymatically active Lp-PLA2 in the sample.

In another aspect of the invention the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample. In another aspect of the invention compound which reduces active thiol(s) in the sample is DTNB.

In another aspect of the invention the sample is incubated at room temperature. In yet another aspect of the invention the sample is incubated at 37°C. In a further aspect the sample is incubated from about 2 to about 120 minutes. In another aspect the sample is incubated from about 5 to about 30 minutes.

In yet another aspect the substrate is selected from the group consisting of:



wherein,

R is selected from the group consisting of: (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>7</sub>CH=CH(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>

Y<sub>1</sub> is selected from the group consisting of: (CO)<sub>1-2</sub> or (CH<sub>2</sub>)<sub>2-7</sub>

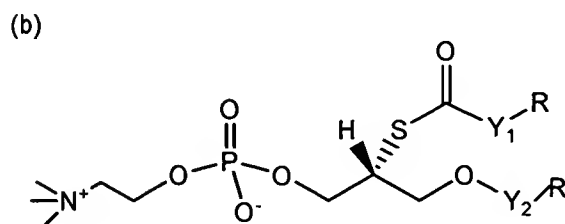
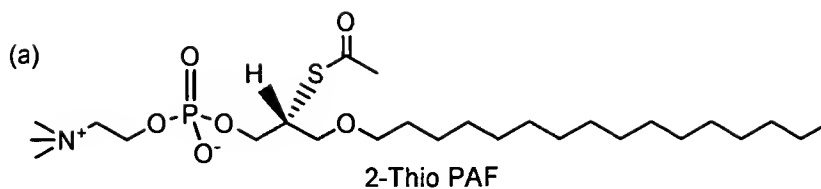
Y<sub>2</sub> is selected from the group consisting of: CO or CH<sub>2</sub>

In another aspect of the invention the substrate is an oxidized derivative of (a) or (b).

The invention further comprises comparing measured free thiol product of step (c) to free thiol product in a control comprising an enzymatically active Lp-PLA2 standard. In a

5 further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system. In a preferred aspect of the invention the method above is conducted in a multi-well plate.

10 The invention is also directed to a kit for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising a compound which reduces active thiol(s) and a substrate converted to a detectable product in the presence of Lp-PLA2. In yet another aspect the substrate is selected from the group consisting of:



wherein,

R is selected from the group consisting of: (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>7</sub>CH=CH(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>

Y<sub>1</sub> is selected from the group consisting of: (CO)<sub>1-2</sub> or (CH<sub>2</sub>)<sub>2-7</sub>

Y<sub>2</sub> is selected from the group consisting of: CO or CH<sub>2</sub>

In another aspect of the invention the substrate is an oxidized derivative of (a) or (b). In yet another aspect of the invention the kit contains an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.

### BRIEF DESCRIPTION OF THE FIGURES

- 10 **Figure 1A and Figure 1B** display schematics of the Hybrid ImmunoCapture Assay;  
**Figure 2** displays plasma Lp-PLA2 Activity in HIC-ThioPAF Assay (2c10 as capturing mAb);  
**Figure 3** display plasma Lp-PLA2 Activity in HIC-ThioPAF Assay (B200.1 as capturing mAb);  
**Figure 4** displays plasma Lp-PLA2 Activity in HIC-ThioPAF Assay (B501.1 as capturing mAb);  
15 **Figure 5** displays plasma Lp-PLA2 Activity in HIC-MNP Assay (2c10 as capturing mAb);  
**Figure 6** displays plasma Lp-PLA2 Activity in Commercial ThioPAF Assay;

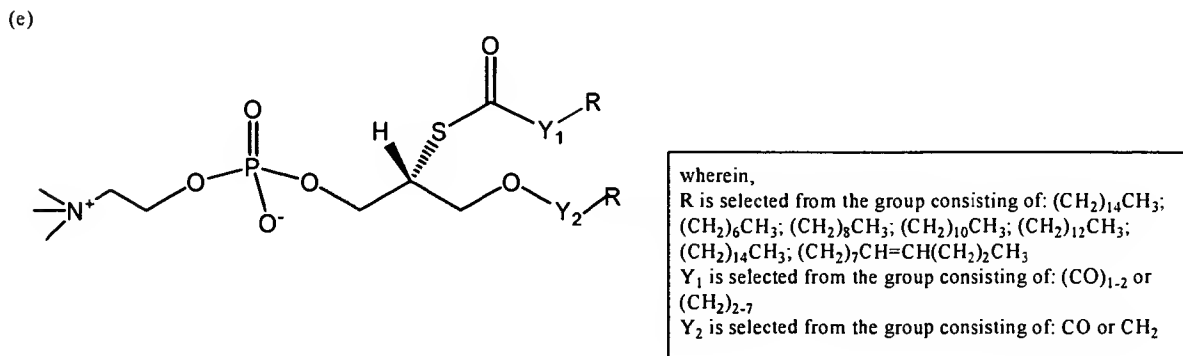
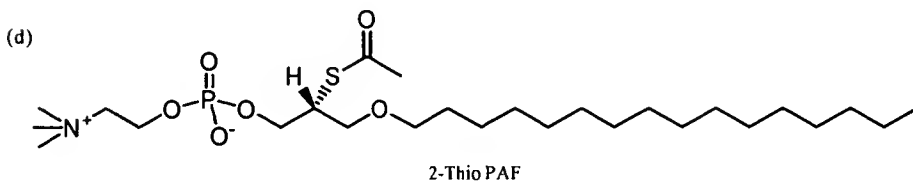
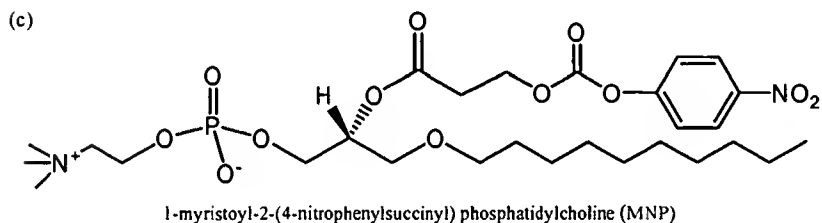
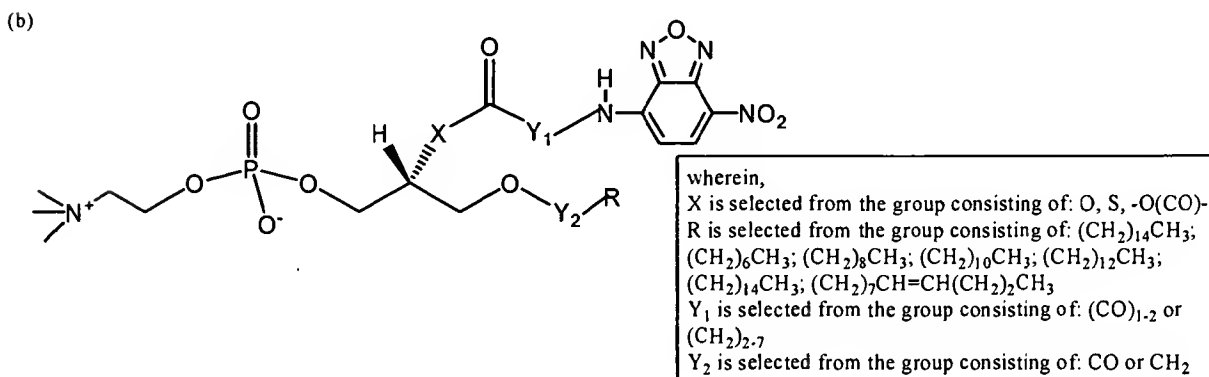
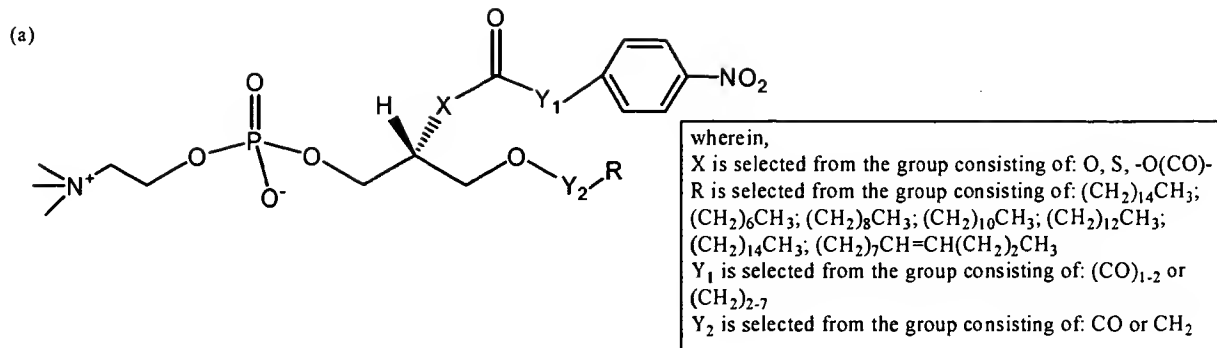
**Figure 7** displays plasma sample background in Improved ThioPAF Assay, with DTNB but w/o substrate added;

**Figure 8** displays plasma Lp-PLA2 Activity post incubation step in Improved ThioPAF Assay.

#### DETAILED DESCRIPTION OF THE INVENTION

5           This method is directed to a method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising contacting an immobilized binder, which specifically binds Lp-PLA2, with the sample; washing the immobilized binder to remove an enzymatically active unbound material or an interfering substance(s); contacting the bound Lp-PLA2 with a substrate converted to a detectable product in the presence of Lp-PLA2; and  
10   measuring detectable product indicative of enzymatically active Lp-PLA2 in the sample. In one aspect of the invention the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample. In another aspect of the invention the immobilized binder is an antibody. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred  
15   aspect of the invention the monoclonal antibody is 2C10, 4B4, B200, B501, 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.

          In another aspect of the invention the enzymatically active unbound material is a phospholipase. In a further aspect of the invention the interfering substance(s) is a free-thiol compound. In yet another aspect of the invention the substrate is selected from the group  
20   consisting of:



In a further aspect of the invention the substrate is an oxidized derivative of (a), (b), (c), (d) or (e) above.

In another aspect of the present invention the detectable product has a radioactive, colorimetric, paramagnetic or fluorescent label. Further, the detectable product is measured  
5 flourimetrically, colormetrically, paramagnetically or via radiation.

A further aspect of the invention comprises comparing the measured detectable product to detectable product in a control comprising an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the  
10 recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system. In another aspect of the present invention the immobilized binder is bound to a multi-well plate, a magnetic bead, or a latex bead.

This invention is also directed to a method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising: contacting an binder, which  
15 specifically binds Lp-PLA2, with the sample to form a binder-Lp-PLA2 complex; immobilizing the binder-Lp-PLA2 complex; washing the immobilized binder-Lp-PLA2 complex to remove an enzymatically active unbound material or an interfering substance(s); contacting the immobilized bound Lp-PLA2 with a substrate converted to a detectable product in the presence of Lp-PLA2; and measuring detectable product indicative of enzymatically  
20 active Lp-PLA2 in the sample. In one aspect of the invention the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample. In another aspect of the invention the binder is an antibody. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the monoclonal antibody is 2C10, 4B4, B200, B501,  
25 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.

In another aspect of the invention the binder-Lp-PLA2 complex is immobilized by binding to an immobilized compound. In a further aspect the immobilized compound is an antibody. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the  
30 monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody.

In another aspect of the invention the immobilized compound is bound to a multi-well plate, a magnetic bead, or a latex bead.

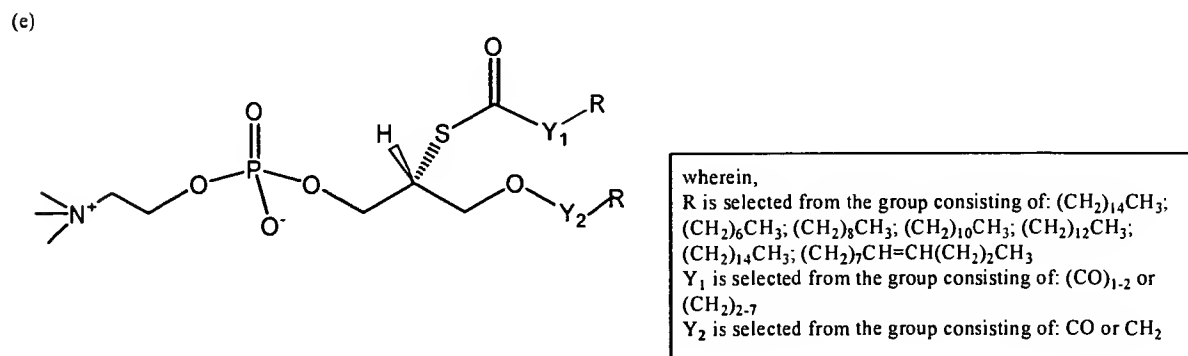
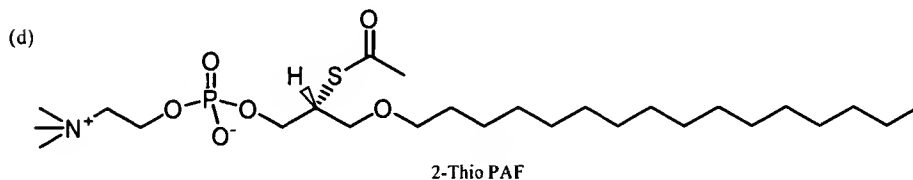
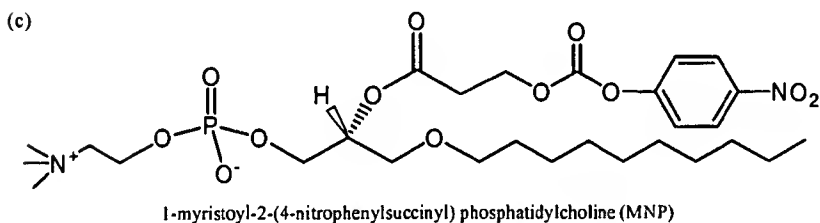
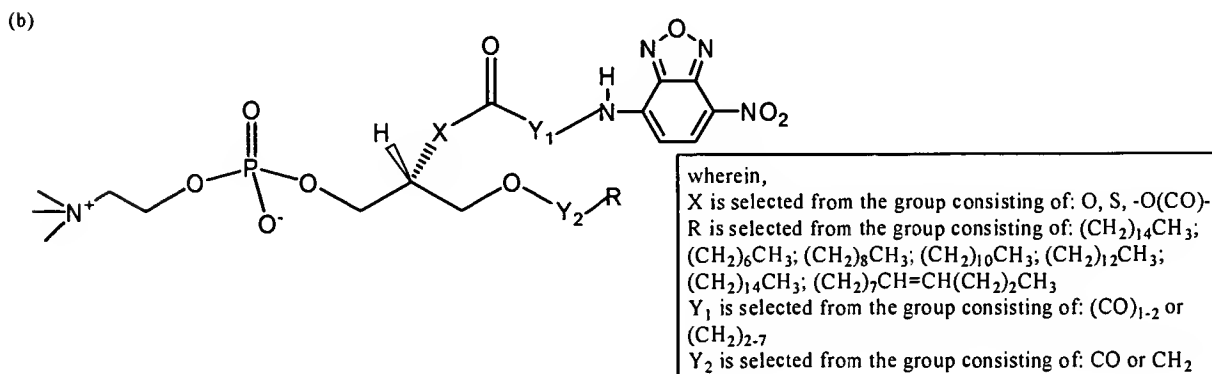
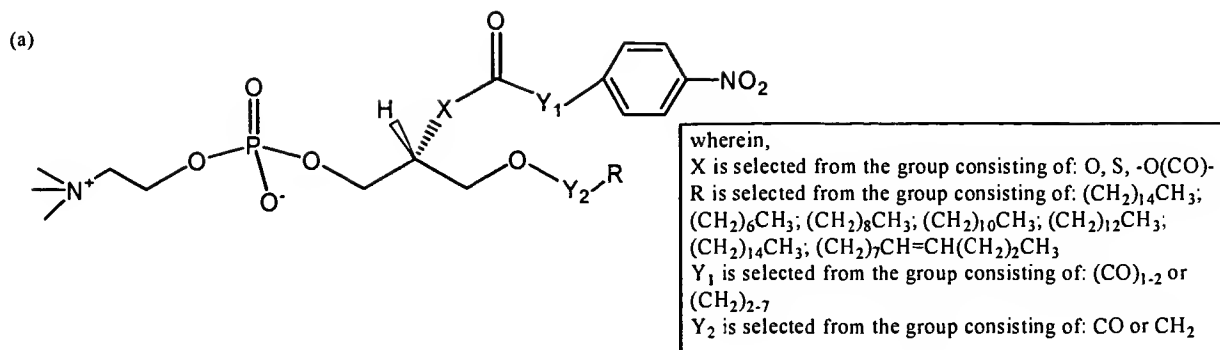
In another aspect of the invention the binder is conjugated to an immobilizing agent. In a further aspect of the invention the binder conjugated to an immobilizing agent is an antibody.

5 . In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the monoclonal antibody is 2C10, 4B4, B200, B501, 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.

In another aspect of the invention the immobilizing agent is an antibody, protein or compound capable of binding an immobilized compound. In preferred aspect of the invention  
10 the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred aspect of the invention the monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody. In another highly preferred aspect the immobilizing agent is biotin.

In a further aspect of the invention the immobilizing agent, conjugated to the binder-Lp-  
15 PLA2 complex, binds to an immobilized compound. In a preferred aspect the immobilized compound is bound to a multi-well plate, a magnetic bead, or a latex bead. In another aspect of the invention the bound compound is an antibody, protein or compound capable of binding the conjugated immobilizing agent. In preferred aspect of the invention the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody. In a highly preferred  
20 aspect of the invention the monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody. . In another highly preferred aspect the immobilizing agent is streptavidin.

In another aspect of the invention the enzymatically active unbound material is a phospholipase. In another aspect of the invention the interfering substance(s) is a free-thiol  
25 compound. In yet another aspect of the invention the substrate is selected from the group consisting of:



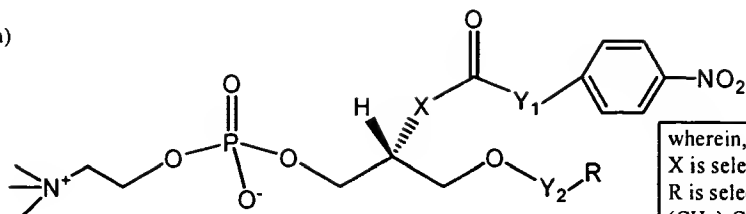
In a further aspect of the invention the substrate is an oxidized derivative of (a), (b), (c), (d) or (e) above.

In another aspect of the present invention the detectable product has a radioactive, colorimetric, paramagnetic or fluorescent label. Further, the detectable product is measured  
5 flourimetrically, colormetrically, paramagnetically or via radiation.

A further aspect of the invention comprises comparing the measured detectable product to detectable product in a control comprising an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the  
10 recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system. In another aspect of the present invention the immobilized binder is bound to a multi-well plate, a magnetic bead, or a latex bead.

The invention is also directed to a kit for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising a binder which specifically binds Lp-  
15 PLA2 and a substrate converted to a detectable product in the presence of Lp-PLA2. In another aspect of the invention the the substrate is selected from the group consisting of:

(a)

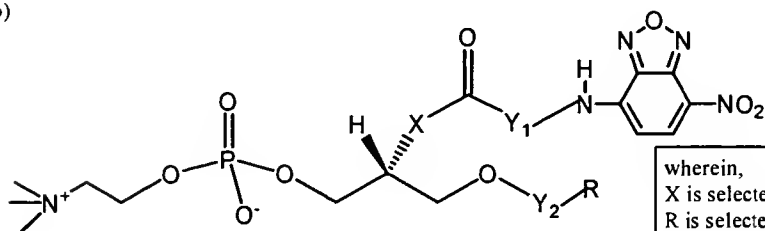


wherein,

X is selected from the group consisting of: O, S, -O(CO)-

R is selected from the group consisting of: (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>;(CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>;(CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>7</sub>CH=CH(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>Y<sub>1</sub> is selected from the group consisting of: (CO)<sub>1-2</sub> or(CH<sub>2</sub>)<sub>2-7</sub>Y<sub>2</sub> is selected from the group consisting of: CO or CH<sub>2</sub>

(b)

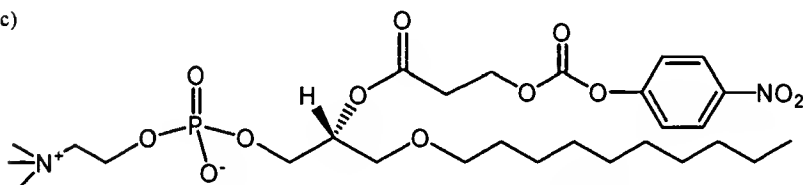


wherein,

X is selected from the group consisting of: O, S, -O(CO)-

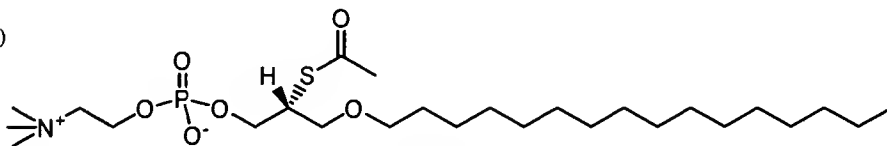
R is selected from the group consisting of: (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>;(CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>;(CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>7</sub>CH=CH(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>Y<sub>1</sub> is selected from the group consisting of: (CO)<sub>1-2</sub> or(CH<sub>2</sub>)<sub>2-7</sub>Y<sub>2</sub> is selected from the group consisting of: CO or CH<sub>2</sub>

(c)



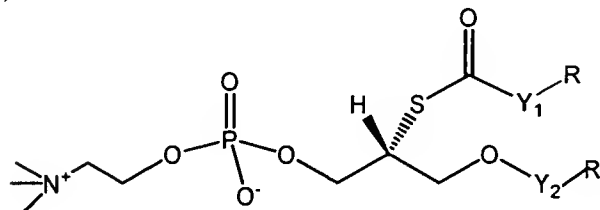
1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine (MNP)

(d)



2-Thio PAF

(e)



wherein,

R is selected from the group consisting of: (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>;(CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>;(CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>7</sub>CH=CH(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>Y<sub>1</sub> is selected from the group consisting of: (CO)<sub>1-2</sub> or(CH<sub>2</sub>)<sub>2-7</sub>Y<sub>2</sub> is selected from the group consisting of: CO or CH<sub>2</sub>

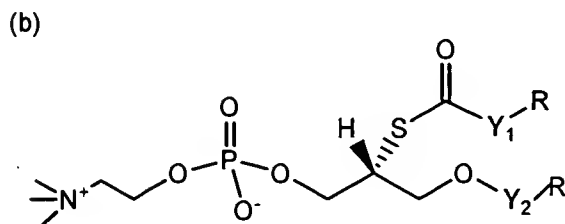
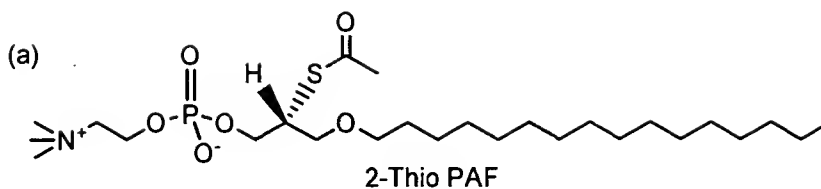
In a further aspect of the invention the substrate is an oxidized derivative of (a), (b), (c), (d) or (e) above. Another aspect of the invention is a kit comprising an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.

The invention is also directed to a method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising: incubating the sample with a compound which reduces active thiol(s) in the sample; contacting the incubated sample with a substrate converted to a free thiol product in the presence of enzymatically active Lp-PLA2; and measuring free thiol product indicative of enzymatically active Lp-PLA2 in the sample.

In another aspect of the invention the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample. In another aspect of the invention compound which reduces active thiol(s) in the sample is DTNB.

In another aspect of the invention the sample is incubated at room temperature. In yet another aspect of the invention the sample is incubated at 37°C. In a further aspect the sample is incubated from about 2 to about 120 minutes. In another aspect the sample is incubated from about 5 to about 30 minutes.

In yet another aspect the substrate is selected from the group consisting of:



wherein,

R is selected from the group consisting of:  $(\text{CH}_2)_{14}\text{CH}_3$ ;  $(\text{CH}_2)_6\text{CH}_3$ ;  $(\text{CH}_2)_8\text{CH}_3$ ;  $(\text{CH}_2)_{10}\text{CH}_3$ ;  $(\text{CH}_2)_{12}\text{CH}_3$ ;  $(\text{CH}_2)_{14}\text{CH}_3$ ;  $(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_2\text{CH}_3$

$\text{Y}_1$  is selected from the group consisting of:  $(\text{CO})_{1-2}$  or  $(\text{CH}_2)_{2-7}$

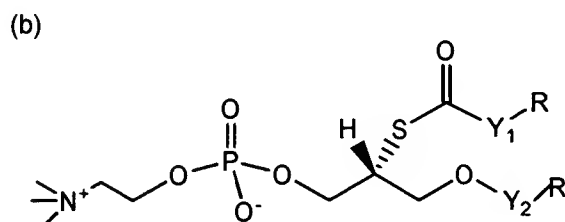
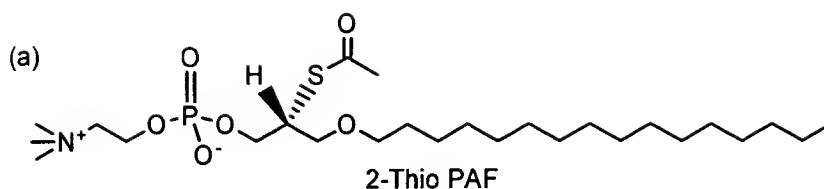
$\text{Y}_2$  is selected from the group consisting of: CO or  $\text{CH}_2$

In another aspect of the invention the substrate is an oxidized derivative of (a) or (b).

The invention further comprises comparing measured free thiol product of step (c) to free thiol product in a control comprising an enzymatically active Lp-PLA2 standard. In a

5 further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system. In a preferred aspect of the invention the method above is conducted in a multi-well plate.

10 The invention is also directed to a kit for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising a compound which reduces active thiol(s) and a substrate converted to a detectable product in the presence of Lp-PLA2. In yet another aspect the substrate is selected from the group consisting of:



wherein,

R is selected from the group consisting of: (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>;

(CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>7</sub>CH=CH(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>

Y<sub>1</sub> is selected from the group consisting of: (CO)<sub>1-2</sub> or (CH<sub>2</sub>)<sub>2-7</sub>

Y<sub>2</sub> is selected from the group consisting of: CO or CH<sub>2</sub>

In another aspect of the invention the substrate is an oxidized derivative of (a) or (b). In yet another aspect of the invention the kit contains an enzymatically active Lp-PLA2 standard. In a further aspect of the invention the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein. In yet a further aspect of the invention the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.

One of ordinary skill in the art will readily recognize additional sources of antibodies exist for the practice of the invention herein. Specifically,

10 An "antibody" refers to an intact immunoglobulin, or to an antigen-binding portion thereof that competes with the intact antibody for specific binding to a molecular species, *e.g.*, a polypeptide of the instant invention. Antigen-binding portions may be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. Antigen-binding portions include, *inter alia*, Fab, Fab', F(ab')<sub>2</sub>, Fv, dAb, and complementarity  
15 determining region (CDR) fragments, single-chain antibodies (scFv), chimeric antibodies, diabodies and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. A Fab fragment is a monovalent fragment consisting of the VL, VH, CL and CH1 domains; a F(ab')<sub>2</sub> fragment is a

bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consists of the VH and CH1 domains; a Fv fragment consists of the VL and VH domains of a single arm of an antibody; and a dAb fragment consists of a VH domain. See, e.g., Ward *et al.*, *Nature* 341: 544-546 (1989).

5 By “bind specifically” and “specific binding” as used herein it is meant the ability of the antibody to bind to a first molecular species in preference to binding to other molecular species with which the antibody and first molecular species are admixed. An antibody is said specifically to “recognize” a first molecular species when it can bind specifically to that first molecular species.

10 A single-chain antibody (scFv) is an antibody in which VL and VH regions are paired to form a monovalent molecule via a synthetic linker that enables them to be made as a single protein chain. See, e.g., Bird *et al.*, *Science* 242: 423-426 (1988); Huston *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 5879-5883 (1988). Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too  
15 short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites. See e.g., Holliger *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993); Poljak *et al.*, *Structure* 2: 1121-1123 (1994). One or more CDRs may be incorporated into a molecule either covalently or noncovalently to make it an immunoadhesin. An immunoadhesin may  
20 incorporate the CDR(s) as part of a larger polypeptide chain, may covalently link the CDR(s) to another polypeptide chain, or may incorporate the CDR(s) noncovalently. The CDRs permit the immunoadhesin to specifically bind to a particular antigen of interest. A chimeric antibody is an antibody that contains one or more regions from one antibody and one or more regions from one or more other antibodies.

25 An antibody may have one or more binding sites. If there is more than one binding site, the binding sites may be identical to one another or may be different. For instance, a naturally occurring immunoglobulin has two identical binding sites, a single-chain antibody or Fab fragment has one binding site, while a “bispecific” or “bifunctional” antibody has two different binding sites.

30 An “isolated antibody” is an antibody that (1) is not associated with naturally-associated components, including other naturally-associated antibodies, that accompany it in its native state, (2) is free of other proteins from the same species, (3) is expressed by a cell from a

different species, or (4) does not occur in nature. It is known that purified proteins, including purified antibodies, may be stabilized with non-naturally-associated components. The non-naturally-associated component may be a protein, such as albumin (*e.g.*, BSA) or a chemical such as polyethylene glycol (PEG).

5           A “neutralizing antibody” or “an inhibitory antibody” is an antibody that inhibits the activity of a polypeptide or blocks the binding of a polypeptide to a ligand that normally binds to it. An “activating antibody” is an antibody that increases the activity of a polypeptide.

          The term “epitope” includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active  
10   surface groupings of molecules such as amino acids or sugar side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics. An antibody is said to specifically bind an antigen when the dissociation constant is less than 1  $\mu$ M, preferably less than 100 nM and most preferably less than 10 nM.

          As is well known in the art, the degree to which an antibody can discriminate as among  
15   molecular species in a mixture will depend, in part, upon the conformational relatedness of the species in the mixture; typically, the antibodies of the present invention will discriminate over adventitious binding to Lp-PLA2 polypeptides by at least two-fold, more typically by at least 5-fold, typically by more than 10-fold, 25-fold, 50-fold, 75-fold, and often by more than 100-fold, and on occasion by more than 500-fold or 1000-fold.

20           Typically, the affinity or avidity of an antibody (or antibody multimer, as in the case of an IgM pentamer) of the present invention for a protein or protein fragment of the present invention will be at least about  $1 \times 10^{-6}$  molar (M), typically at least about  $5 \times 10^{-7}$  M,  $1 \times 10^{-7}$  M, with affinities and avidities of at least  $1 \times 10^{-8}$  M,  $5 \times 10^{-9}$  M,  $1 \times 10^{-10}$  M and up to  $1 \times 10^{-13}$  M proving especially useful.

25           The antibodies of the present invention can be naturally occurring forms, such as IgG, IgM, IgD, IgE, IgY, and IgA, from any avian, reptilian, or mammalian species.

          IgG, IgM, IgD, IgE, IgY, and IgA antibodies of the present invention are also usefully obtained from other species, including mammals such as rodents (typically mouse, but also rat, guinea pig, and hamster), lagomorphs (typically rabbits), and also larger mammals, such as  
30   sheep, goats, cows, and horses; or egg laying birds or reptiles such as chickens or alligators. In such cases, as with the transgenic human-antibody-producing non-human mammals, fortuitous immunization is not required, and the non-human mammal is typically affirmatively

immunized, according to standard immunization protocols, with the polypeptide of the present invention. One form of avian antibodies may be generated using techniques described in WO 00/29444, published 25 May 2000.

As discussed above, virtually all fragments of 8 or more contiguous amino acids of a polypeptide of the present invention can be used effectively as immunogens when conjugated to a carrier, typically a protein such as bovine thyroglobulin, keyhole limpet hemocyanin, or bovine serum albumin, conveniently using a bifunctional linker such as those described elsewhere above, which discussion is incorporated by reference here.

Immunogenicity can also be conferred by fusion of the polypeptide of the present invention to other moieties. For example, polypeptides of the present invention can be produced by solid phase synthesis on a branched polylysine core matrix; these multiple antigenic peptides (MAPs) provide high purity, increased avidity, accurate chemical definition and improved safety in vaccine development. Tam *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 5409-5413 (1988); Posnett *et al.*, *J. Biol. Chem.* 263: 1719-1725 (1988).

Protocols for immunizing non-human mammals or avian species are well-established in the art. See Harlow *et al.* (eds.), Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1998); Coligan *et al.* (eds.), Current Protocols in Immunology, John Wiley & Sons, Inc. (2001); Zola, Monoclonal Antibodies: Preparation and Use of Monoclonal Antibodies and Engineered Antibody Derivatives (Basics: From Background to Bench), Springer Verlag (2000); Gross M, Speck *J.Dtsch. Tierarztl. Wochenschr.* 103: 417-422 (1996). Immunization protocols often include multiple immunizations, either with or without adjuvants such as Freund's complete adjuvant and Freund's incomplete adjuvant, and may include naked DNA immunization (Moss, *Semin. Immunol.* 2: 317-327 (1990)).

Antibodies from non-human mammals and avian species can be polyclonal or monoclonal, with polyclonal antibodies having certain advantages in immunohistochemical detection of the polypeptides of the present invention and monoclonal antibodies having advantages in identifying and distinguishing particular epitopes of the polypeptides of the present invention. Antibodies from avian species may have particular advantage in detection of the polypeptides of the present invention, in human serum or tissues (Vikinge *et al.*, *Biosens. Bioelectron.* 13: 1257-1262 (1998)). Following immunization, the antibodies of the present invention can be obtained using any art-accepted technique. Such techniques are well known in the art and are described in detail in references such as Coligan, *supra*; Zola, *supra*; Howard

*et al.* (eds.), Basic Methods in Antibody Production and Characterization, CRC Press (2000); Harlow, *supra*; Davis (ed.), Monoclonal Antibody Protocols, Vol. 45, Humana Press (1995); Delves (ed.), Antibody Production: Essential Techniques, John Wiley & Son Ltd (1997); and Kenney, Antibody Solution: An Antibody Methods Manual, Chapman & Hall (1997).

5 Briefly, such techniques include, *inter alia*, production of monoclonal antibodies by hybridomas and expression of antibodies or fragments or derivatives thereof from host cells engineered to express immunoglobulin genes or fragments thereof. These two methods of production are not mutually exclusive: genes encoding antibodies specific for the polypeptides of the present invention can be cloned from hybridomas and thereafter expressed in other host  
10 cells. Nor need the two necessarily be performed together: *e.g.*, genes encoding antibodies specific for the polypeptides of the present invention can be cloned directly from B cells known to be specific for the desired protein, as further described in U.S. Patent No. 5,627,052, the disclosure of which is incorporated herein by reference in its entirety, or from antibody-displaying phage.

15 Recombinant expression in host cells is particularly useful when fragments or derivatives of the antibodies of the present invention are desired.

Host cells for recombinant antibody production of whole antibodies, antibody fragments, or antibody derivatives can be prokaryotic or eukaryotic.

20 Prokaryotic hosts are particularly useful for producing phage displayed antibodies of the present invention.

The technology of phage-displayed antibodies, in which antibody variable region fragments are fused, for example, to the gene III protein (pIII) or gene VIII protein (pVIII) for display on the surface of filamentous phage, such as M13, is by now well-established. *See, e.g.*, Sidhu, *Curr. Opin. Biotechnol.* 11(6): 610-6 (2000); Griffiths *et al.*, *Curr. Opin. Biotechnol.* 9(1): 102-8 (1998); Hoogenboom *et al.*, *Immunotechnology*, 4(1): 1-20 (1998); Rader *et al.*, *Current Opinion in Biotechnology* 8: 503-508 (1997); Aujame *et al.*, *Human Antibodies* 8: 155-168 (1997); Hoogenboom, *Trends in Biotechnol.* 15: 62-70 (1997); de Kruif *et al.*, 17: 453-455 (1996); Barbas *et al.*, *Trends in Biotechnol.* 14: 230-234 (1996); Winter *et al.*, *Ann. Rev. Immunol.* 433-455 (1994). Techniques and protocols required to generate,  
30 propagate, screen (pan), and use the antibody fragments from such libraries have recently been compiled. *See, e.g.*, Barbas (2001), *supra*; Kay, *supra*; and Abelson, *supra*.

Typically, phage-displayed antibody fragments are scFv fragments or Fab fragments; when desired, full length antibodies can be produced by cloning the variable regions from the displaying phage into a complete antibody and expressing the full length antibody in a further prokaryotic or a eukaryotic host cell. Eukaryotic cells are also useful for expression of the antibodies, antibody fragments, and antibody derivatives of the present invention. For example, antibody fragments of the present invention can be produced in *Pichia pastoris* and in *Saccharomyces cerevisiae*. See, e.g., Takahashi *et al.*, *Biosci. Biotechnol. Biochem.* 64(10): 2138-44 (2000); Freyre *et al.*, *J. Biotechnol.* 76(2-3):1 57-63 (2000); Fischer *et al.*, *Biotechnol. Appl. Biochem.* 30 (Pt 2): 117-20 (1999); Pennell *et al.*, *Res. Immunol.* 149(6): 599-603 (1998); Eldin *et al.*, *J. Immunol. Methods.* 201(1): 67-75 (1997);, Frenken *et al.*, *Res. Immunol.* 149(6): 589-99 (1998); and Shusta *et al.*, *Nature Biotechnol.* 16(8): 773-7 (1998).

Antibodies, including antibody fragments and derivatives, of the present invention can also be produced in insect cells. See, e.g., Li *et al.*, *Protein Expr. Purif.* 21(1): 121-8 (2001); Ailor *et al.*, *Biotechnol. Bioeng.* 58(2-3): 196-203 (1998); Hsu *et al.*, *Biotechnol. Prog.* 13(1): 96-104 (1997); Edelman *et al.*, *Immunology* 91(1): 13-9 (1997); and Nesbit *et al.*, *J. Immunol. Methods* 151(1-2): 201-8 (1992).

Antibodies and fragments and derivatives thereof of the present invention can also be produced in plant cells, particularly maize or tobacco, Giddings *et al.*, *Nature Biotechnol.* 18(11): 1151-5 (2000); Gavilondo *et al.*, *Biotechniques* 29(1): 128-38 (2000); Fischer *et al.*, *J. Biol. Regul. Homeost. Agents* 14(2): 83-92 (2000); Fischer *et al.*, *Biotechnol. Appl. Biochem.* 30 (Pt 2): 113-6 (1999); Fischer *et al.*, *Biol. Chem.* 380(7-8): 825-39 (1999); Russell, *Curr. Top. Microbiol. Immunol.* 240: 119-38 (1999); and Ma *et al.*, *Plant Physiol.* 109(2): 341-6 (1995).

Antibodies, including antibody fragments and derivatives, of the present invention can also be produced in transgenic, non-human, mammalian milk. See, e.g. Pollock *et al.*, *J. Immunol Methods.* 231: 147-57 (1999); Young *et al.*, *Res. Immunol.* 149: 609-10 (1998); and Limonta *et al.*, *Immunotechnology* 1: 107-13 (1995).

Mammalian cells useful for recombinant expression of antibodies, antibody fragments, and antibody derivatives of the present invention include CHO cells, COS cells, 293 cells, and myeloma cells. Verma *et al.*, *J. Immunol. Methods* 216(1-2):165-81 (1998) review and compare bacterial, yeast, insect and mammalian expression systems for expression of antibodies. Antibodies of the present invention can also be prepared by cell free translation, as

further described in Merk *et al.*, *J. Biochem.* (Tokyo) 125(2): 328-33 (1999) and Ryabova *et al.*, *Nature Biotechnol.* 15(1): 79-84 (1997), and in the milk of transgenic animals, as further described in Pollock *et al.*, *J. Immunol. Methods* 231(1-2): 147-57 (1999).

The invention further provides antibody fragments that bind specifically to one or more  
5 of the polypeptides of the present invention, to one or more of the polypeptides encoded by the  
isolated nucleic acid molecules of the present invention, or the binding of which can be  
competitively inhibited by one or more of the polypeptides of the present invention or one or  
more of the polypeptides encoded by the isolated nucleic acid molecules of the present  
invention. Among such useful fragments are Fab, Fab', Fv, F(ab)'<sub>2</sub>, and single chain Fv (scFv)  
10 fragments. Other useful fragments are described in Hudson, *Curr. Opin. Biotechnol.* 9(4):  
395-402 (1998).

The present invention also relates to antibody derivatives that bind specifically to one or  
more of the polypeptides of the present invention, to one or more of the polypeptides encoded  
by the isolated nucleic acid molecules of the present invention, or the binding of which can be  
15 competitively inhibited by one or more of the polypeptides of the present invention or one or  
more of the polypeptides encoded by the isolated nucleic acid molecules of the present  
invention.

Among such useful derivatives are chimeric, primatized, and humanized antibodies;  
such derivatives are less immunogenic in human beings, and thus are more suitable for *in vivo*  
20 administration, than are unmodified antibodies from non-human mammalian species. Another  
useful method is PEGylation to increase the serum half life of the antibodies.

Chimeric antibodies typically include heavy and/or light chain variable regions  
(including both CDR and framework residues) of immunoglobulins of one species, typically  
mouse, fused to constant regions of another species, typically human. See, e.g., Morrison *et al.*,  
25 *Proc. Natl. Acad. Sci USA* 81(21): 6851-5 (1984); Sharon *et al.*, *Nature* 309(5966): 364-7  
(1984); Takeda *et al.*, *Nature* 314(6010): 452-4 (1985); and U.S. Patent No. 5,807,715 the  
disclosure of which is incorporated herein by reference in its entirety. Primatized and  
humanized antibodies typically include heavy and/or light chain CDRs from a murine antibody  
grafted into a non-human primate or human antibody V region framework, usually further  
30 comprising a human constant region, Riechmann *et al.*, *Nature* 332(6162): 323-7 (1988); Co *et al.*,  
*Nature* 351(6326): 501-2 (1991); and U.S. Patent Nos. 6,054,297; 5,821,337; 5,770,196;  
5,766,886; 5,821,123; 5,869,619; 6,180,377; 6,013,256; 5,693,761; and 6,180,370, the

disclosures of which are incorporated herein by reference in their entireties. Other useful antibody derivatives of the invention include heteromeric antibody complexes and antibody fusions, such as diabodies (bispecific antibodies), single-chain diabodies, and intrabodies.

Typical substrates for production and deposition of visually detectable products include  
5 o-nitrophenyl-beta-D-galactopyranoside (ONPG); o-phenylenediamine dihydrochloride (OPD);  
p-nitrophenyl phosphate (PNPP); p-nitrophenyl-beta-D-galactopyranoside (PNPG); 3',3'-  
diaminobenzidine (DAB); 3-amino-9-ethylcarbazole (AEC); 4-chloro-1-naphthol (CN);  
5-bromo-4-chloro-3-indolyl-phosphate (BCIP); ABTS®; BluoGal; iodonitrotetrazolium (INT);  
nitroblue tetrazolium chloride (NBT); phenazine methosulfate (PMS); phenolphthalein  
10 monophosphate (PMP); tetramethyl benzidine (TMB); tetranitroblue tetrazolium (TNBT);  
X-Gal; X-Gluc; and X-Glucoside.

Other substrates can be used to produce products for local deposition that are luminescent. For example, in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), horseradish peroxidase (HRP) can catalyze the oxidation of cyclic diacylhydrazides, such as luminol.  
15 Immediately following the oxidation, the luminol is in an excited state (intermediate reaction product), which decays to the ground state by emitting light. Strong enhancement of the light emission is produced by enhancers, such as phenolic compounds. Advantages include high sensitivity, high resolution, and rapid detection without radioactivity and requiring only small amounts of antibody. *See, e.g., Thorpe et al., Methods Enzymol.* 133: 331-53 (1986); Kricka *et al., J. Immunoassay* 17(1): 67-83 (1996); and Lundqvist *et al., J. Biolumin. Chemilumin.* 10(6):  
20 353-9 (1995). Kits for such enhanced chemiluminescent detection (ECL) are available commercially. The antibodies can also be labeled using colloidal gold.

As another example, when the antibodies of the present invention are used, *e.g.,* for flow cytometric detection, for scanning laser cytometric detection, or for fluorescent  
25 immunoassay, they can usefully be labeled with fluorophores. There are a wide variety of fluorophore labels that can usefully be attached to the antibodies of the present invention. For flow cytometric applications, both for extracellular detection and for intracellular detection, common useful fluorophores can be fluorescein isothiocyanate (FITC), allophycocyanin (APC), R-phycoerythrin (PE), peridinin chlorophyll protein (PerCP), Texas Red, Cy3, Cy5,  
30 fluorescence resonance energy tandem fluorophores such as PerCP-Cy5.5, PE-Cy5, PE-Cy5.5, PE-Cy7, PE-Texas Red, and APC-Cy7.

Other fluorophores include, *inter alia*, Alexa Fluor® 350, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 647 (monoclonal antibody labeling kits available from Molecular Probes, Inc., Eugene, OR, USA), BODIPY dyes, such as BODIPY 493/503, BODIPY FL, BODIPY R6G, BODIPY 530/550, BODIPY TMR, BODIPY 558/568, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, BODIPY TR, BODIPY 630/650, BODIPY 650/665, Cascade Blue, Cascade Yellow, Dansyl, lissamine rhodamine B, Marina Blue, Oregon Green 488, Oregon Green 514, Pacific Blue, rhodamine 6G, rhodamine green, rhodamine red, tetramethylrhodamine, Texas Red (available from Molecular Probes, Inc., Eugene, OR, USA), and Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, all of which are also useful for fluorescently labeling the antibodies of the present invention. For secondary detection using labeled avidin, streptavidin, captavidin or neutravidin, the antibodies of the present invention can usefully be labeled with biotin.

When the antibodies of the present invention are used, *e.g.*, for western blotting applications, they can usefully be labeled with radioisotopes, such as  $^{33}\text{P}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ , and  $^{125}\text{I}$ . As another example, when the antibodies of the present invention are used for radioimmunotherapy, the label can usefully be  $^3\text{H}$ ,  $^{228}\text{Th}$ ,  $^{227}\text{Ac}$ ,  $^{225}\text{Ac}$ ,  $^{223}\text{Ra}$ ,  $^{213}\text{Bi}$ ,  $^{212}\text{Pb}$ ,  $^{212}\text{Bi}$ ,  $^{211}\text{At}$ ,  $^{203}\text{Pb}$ ,  $^{194}\text{Os}$ ,  $^{188}\text{Re}$ ,  $^{186}\text{Re}$ ,  $^{153}\text{Sm}$ ,  $^{149}\text{Tb}$ ,  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{111}\text{In}$ ,  $^{105}\text{Rh}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{97}\text{Ru}$ ,  $^{90}\text{Y}$ ,  $^{90}\text{Sr}$ ,  $^{88}\text{Y}$ ,  $^{72}\text{Se}$ ,  $^{67}\text{Cu}$ , or  $^{47}\text{Sc}$ .

## EXAMPLES

### Example 1: Hybrid ImmunoCapture (HIC) assay

EGTA, NaCl, HEPES, Ellman's reagent; 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), Tris-HCL were obtained from Sigma (St. Louis, MO). Bovine serum albumin was obtained from GIBCO-Invitrogen (Carlsbad, CA). Microtiter plates were obtained from VWR (West Chester, PA). TBS and SuperBlock/TBS Blocking Solution were obtained from Pierce (Rockford, IL). Citric acid monohydrate buffer was obtained from Teknova (Half Moon Bay, CA). 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine (MNP) was obtained from KARLAN (Santa Rosa, CA) and 2-Thio-PAF was obtained from Cayman Chemical (Ann Arbor, MI). Active mammalian recombinant Lp-PLA2 was generated at diaDexus.

## Methods

### Hybrid ImmunoCapture Lp-PLA<sub>2</sub> Activity Assays

A schematic of the Hybrid ImmunoCapture Assay (HIC) is shown in FIG. 1A and FIG. 1B. FIG. 1A shows one embodiment with a direct readout of substrate being converted to product. FIG. 1B shows a secondary readout where the product from the first reaction reacts to form a second product. The HIC-ThioPAF assay is an example of the FIG. 1B embodiment.

Prior to the assay, ethanolic solution of 2-thio PAF was evaporated to dryness under a gentle stream of nitrogen, and reconstituted in 1x Assay Buffer (0.1 M Tris-HCl, pH 7.2, 1mM EGTA) to a final concentration of 400uM. 10 mM DTNB solution was prepared with 0.4M Tri-HCl, pH 7.2. R2 Buffer containing 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine was made by mixing 20mM citric acid monohydrate buffer, pH4.5, and 90mM 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine) in the proportion of 79:1 before assay.

First, microtiter plate was coated with 200 uL of anti Lp-PLA<sub>2</sub> mAb 2C10 (5µg/ml) in 1XTBS and incubated at 4°C, overnight. Then the plate was blocked with 250 µL of SuperBlock TBS Blocking Solution, incubated with shaking at 180rpm at room temperature for 20 min. The plate was washed prior to use.

### *Lp-PLA<sub>2</sub> HIC assay using 2-thio PAF substrate*

First, 170 µL of 1xTBS pH 7.4 buffer was added to a well followed by 30µL of sample, or standard (mammalian recombinant Lp-PLA<sub>2</sub>, lot at 0, 25, 50, 100, 200, 400 ng/ml), and incubated with shaking at room temperature for 1 hr. After the incubation, the plate was washed once with 360µL of Wash Buffer (1xTBS, 0.05% Tween20). Then 160µL of 1x Assay Buffer (0.1M Tris-HCl, pH 7.2, 1mM EGTA), 10µL of DTNB solution (10mM in 0.4M Tris-HCl, pH 7.2), and 55µL of 400 uM 2-thio PAF substrate solution were added to each well, and read at 414 nm in a plate reader (Molecular Device, Sunnyvale, CA) running in kinetic mode (one reading per min), with auto mix on at 37°C, for 5 min. The slope of the curve corresponding to  $\Delta OD^{414}/\text{min}$  was calculated for all standards and samples. The level of Lp-PLA<sub>2</sub> activity in ng/mL was calculated from a standard curve of the slope of rLp-PLA<sub>2</sub> standards. The results are shown in FIG. 2. FIG 3 shows the results of the HIC-ThioPAF assay

with the antibody B200.1 as the capture antibody. FIG 4 shows the results of the HIC-ThioPAF assay with the antibody B501.1 as the capture antibody.

*Lp-PLA2 HIC assay using 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine (MNP) substrate*

First, 190 $\mu$ L of 1xTBS pH 7.4 buffer was added to a well followed by 10 $\mu$ L of sample, or standard (mammalian recombinant Lp-PLA2, at 0, 25, 50, 100, 200, 400 ng/ml), and incubated with shaking at room temperature for 1 hr. After the incubation, the plate was washed once with 360 $\mu$ L of Wash Buffer (1xTBS, 0.05% Tween20). Then 150 $\mu$ L of R1 buffer (100mM HEPES buffer, 150mM NaCl, 5mM EGTA, pH 7.4), and 50 $\mu$ L of freshly made R2 buffer (see above) were added to each well, and read at 405nm in a plate reader (Molecular Device, Sunnyvale, CA) running in kinetic mode (one reading per min), with auto mix on, for 5 min. The slope of the curve corresponding to  $\Delta OD^{405}/\text{min}$  was calculated for all standards and samples. The level of Lp-PLA2 activity in ng/mL was calculated from a standard curve of the slope of rLp-PLA2 standards. FIG. 5 shows the results of the HIC- 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine assay (HIC-MNP) assay.

**Example 2: Improved ThioPAF assay**

FIG. 6 shows the results of the commercially available ThioPAF assay, available from Cayman Chemicals following the manufacturers protocol. Specifically, in that protocol, the DTNB is added concurrently with 2-thio PAF.

Prior to performing the improved assay, ethanolic solution of 2-thio PAF was evaporated to dryness under a gentle stream of nitrogen, and reconstituted in 1x Assay Buffer (0.1 M Tris-HCl, pH 7.2, 1mM EGTA) to a final concentration of 400 $\mu$ M. DTNB solution was prepared with 0.4M Tris-HCl, pH 7.2 to achieve a final concentration of 10mM (4mg DTNB in 1ml buffer).

*Lp-PLA2 activity assay using 2-thio PAF substrate*

In the assay, 83  $\mu$ L of 1x Assay Buffer was mixed with 20  $\mu$ L of sample, or standard (recombinant mammalian Lp-PLA2 at 800, 400, 200, 100, 50, 25, and 0 ng/mL), and 10 $\mu$ L of

the 10mM DTNB solution (in 0.4M Tris-HCl, pH 7.2), and incubated at room temperature for 15 min. FIG. 7 shows the results. After preincubation with DTNB to eliminate background signal from free thiol(s), 112 $\mu$ l of the 400 $\mu$ M 2-thio PAF solution was added and the plate was read at 414 nm in a plate reader (Molecular Device, Sunnyvale, CA) running in kinetic mode (one reading per min), with auto mix off at room temperature, for 5 min. The slope of the curve corresponding to  $\Delta OD^{414}/\text{mmin}$  was calculated for all standards and samples. The level of Lp-PLA2 activity in ng/mL was calculated from a standard curve of the slope of rLp-PLA2 standards. The results are shown in FIG. 8.

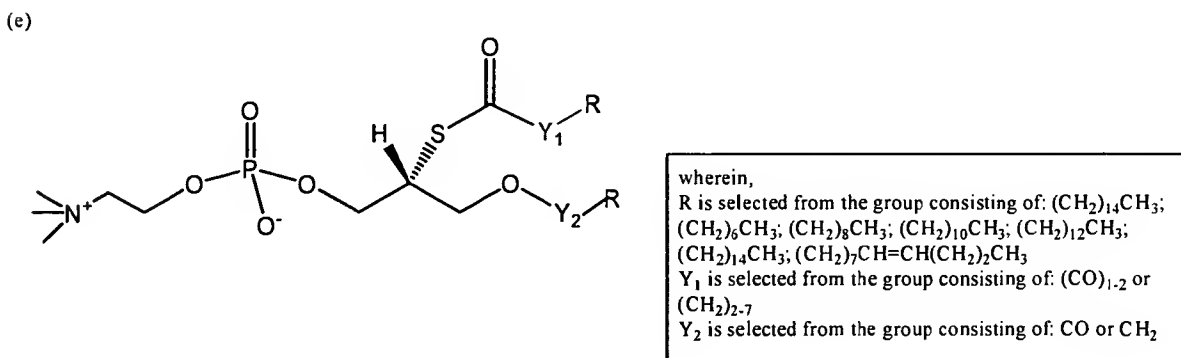
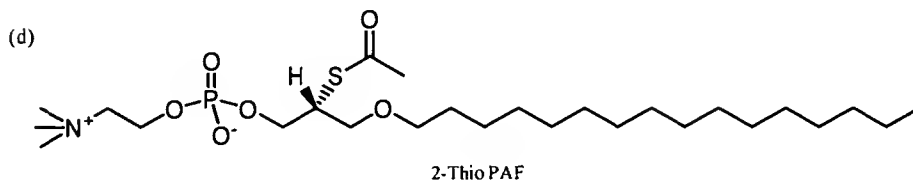
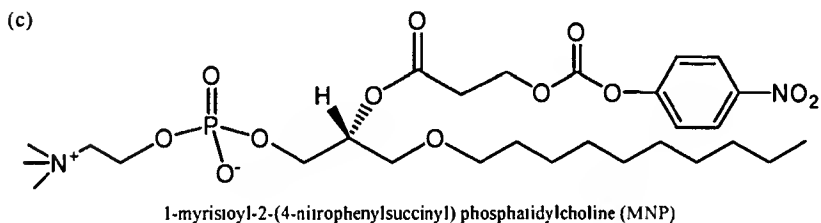
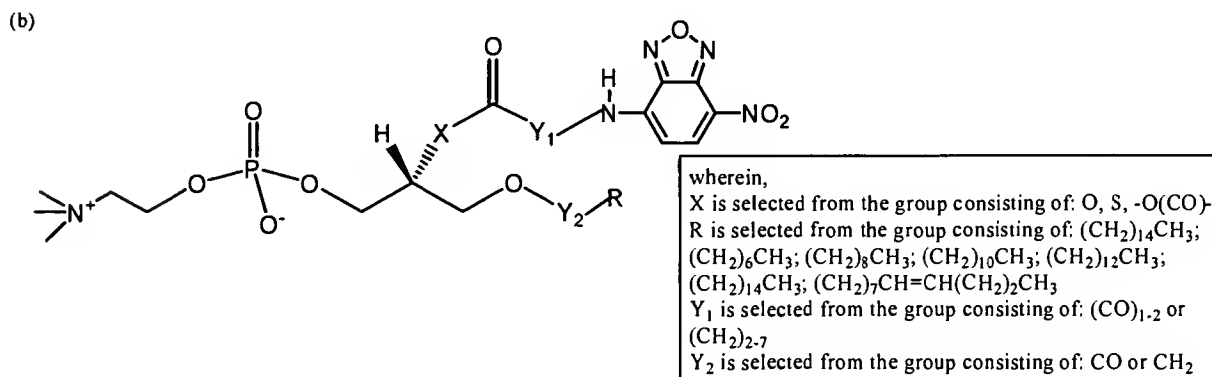
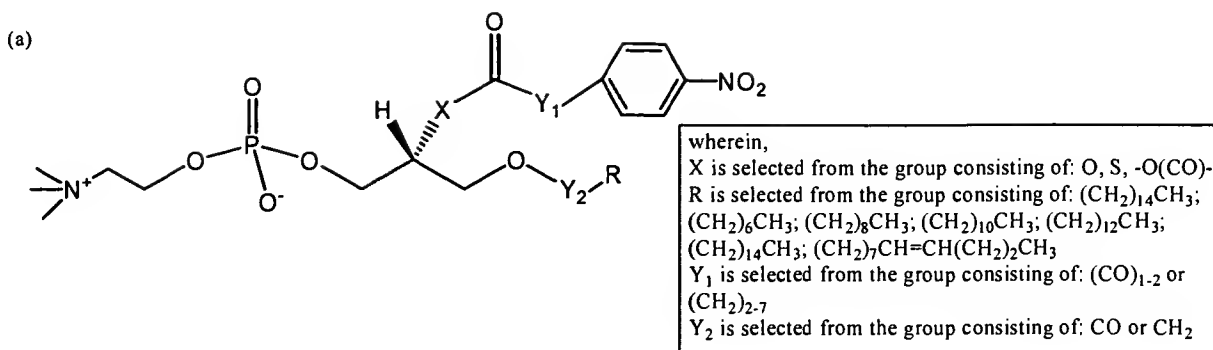
The table below shows the level of Lp-PLA2 activity in ng/mL for a set of 24 diverse plasma samples, determined twice per assay (Commercial Thio-PAF and Improved Thio-PAF). In addition to the activity values, the Coefficient of Variations (CVs) is reported for each sample per assay and an overall average for each assay. Using the Improved Thio-PAF assay improved the CVs from an average of 10.7% to 4.5%.

Sample ID	Comm. Thio-PAF result #1	Comm. Thio-PAF result #2	CV Comm. Thio-PAF Assay	Improved Thio-PAF result #1	Improved Thio-PAF result #2	CV Improved Thio-PAF
1	387.807	544.836	16.8%	212.993	229.352	3.7%
2	326.023	471.18	18.2%	180.75	192.002	3.0%
3	269.221	283.475	2.6%	174.951	189.252	3.9%
4	251.099	250.848	0.1%	133.483	136.896	1.3%
5	341.257	236.061	18.2%	186.841	215.285	7.1%
6	374.072	346.048	3.9%	174.088	182.849	2.5%
7	468.923	429.38	4.4%	209.196	231.258	5.0%
8	447.66	324.269	16.0%	169.298	193.695	6.7%
9	209.819	287.641	15.6%	144.495	165.483	6.8%
10	373.44	385.316	1.6%	180.249	216.328	9.1%
11	337.737	335.674	0.3%	189.426	200.751	2.9%
12	265.701	204.651	13.0%	128.509	144.265	5.8%
13	317.323	349.698	4.9%	166.692	187.637	5.9%
14	216.858	243.403	5.8%	119.438	148.434	10.8%
15	406.76	410.396	0.4%	184.451	205.195	5.3%
16	213.429	232.885	4.4%	138.28	136.004	0.8%
17	237.182	318.948	14.7%	145.778	148.521	0.9%
18	198.898	262.459	13.8%	135.28	150.828	5.4%

19	254.131	383.769	20.3%	153.296	176.586	7.1%
20	213.483	329.673	21.4%	109.556	119.442	4.3%
21	238.446	342.15	17.9%	150.295	172.425	6.9%
22	225.685	275.164	9.9%	158.967	164.756	1.8%
23	246.947	357.185	18.2%	137.172	136.212	0.4%
24	228.32	300.263	13.6%	153.578	155.559	0.6%
Ave. CV			10.7%			4.5%

## WE CLAIM:

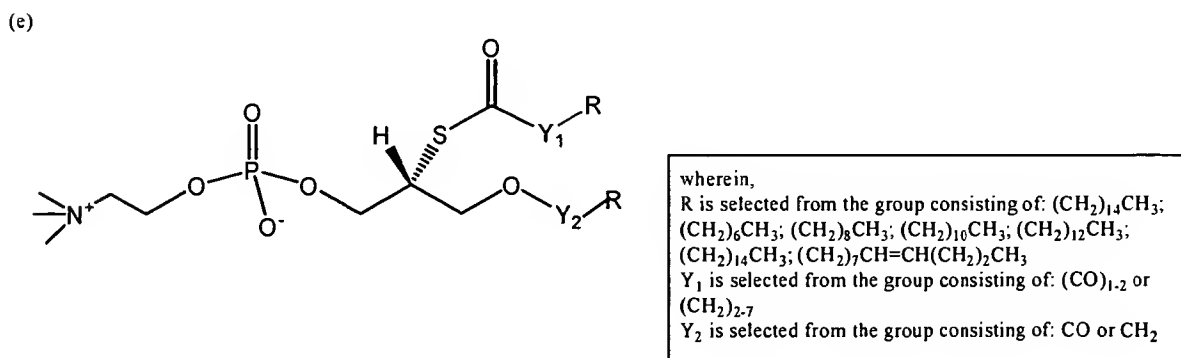
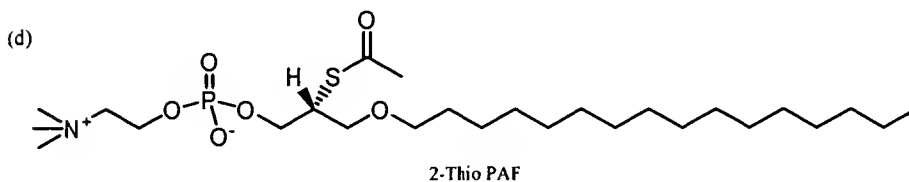
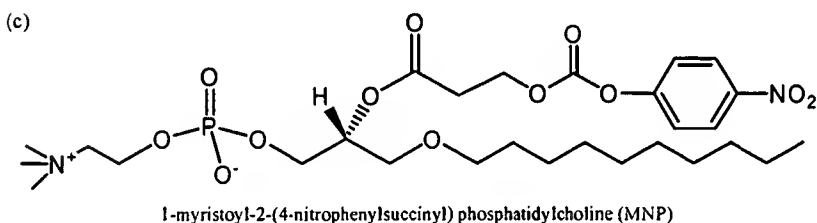
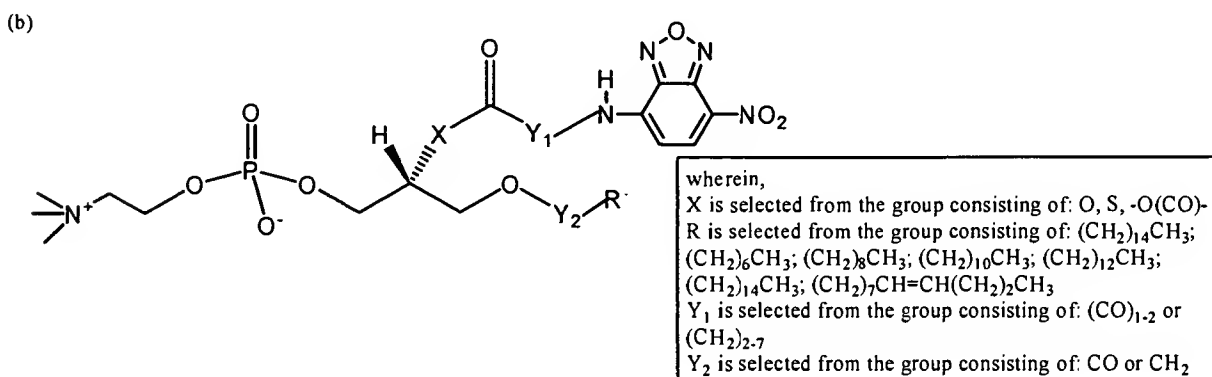
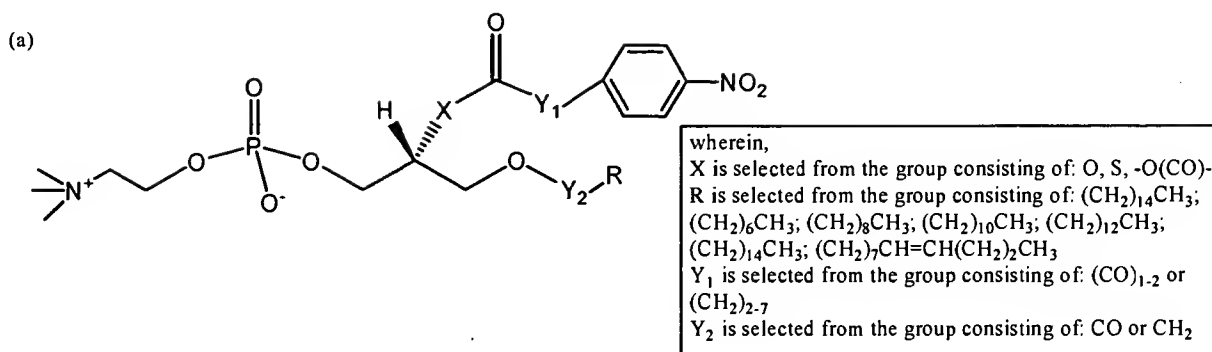
1. A method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising:
  - (a) contacting an immobilized binder, which specifically binds Lp-PLA2,  
5 with the sample;
  - (b) washing the immobilized binder to remove an enzymatically active unbound material or an interfering substance(s);
  - (c) contacting the bound Lp-PLA2 with a substrate converted to a detectable product in the presence of Lp-PLA2; and
  - 10 (d) measuring detectable product indicative of enzymatically active Lp-PLA2 in the sample.
2. The method of claim 1, wherein the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample.
3. The method of claim 1, wherein the immobilized binder is an antibody.
- 15 4. The method of claim 3, wherein the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody.
5. The method of claim 4, wherein the monoclonal antibody is 2C10, 4B4, B200, B501, 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.
6. The method of claim 1 wherein the enzymatically active unbound material is a  
20 phospholipase.
7. The method of claim 1 wherein the interfering substance(s) is a free-thiol compound.
8. The method of claim 1, wherein the substrate is selected from the group consisting of:



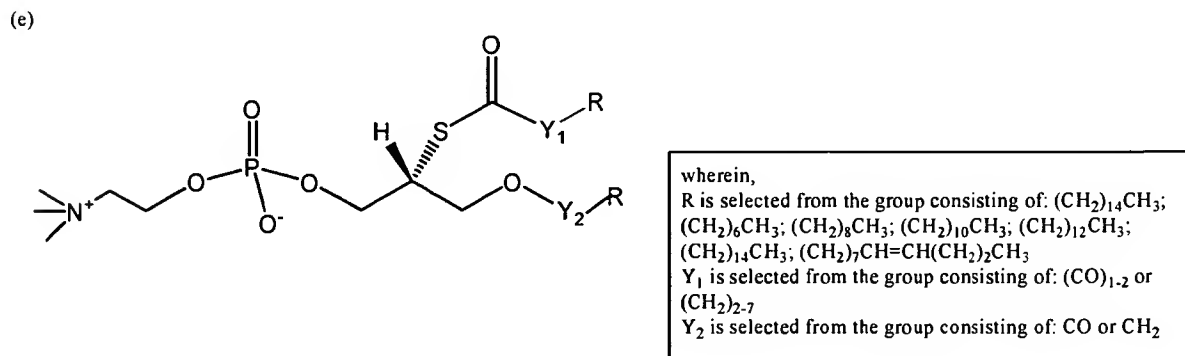
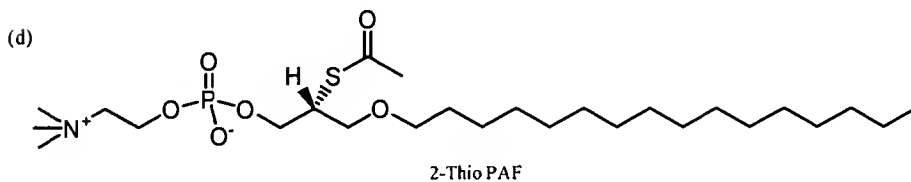
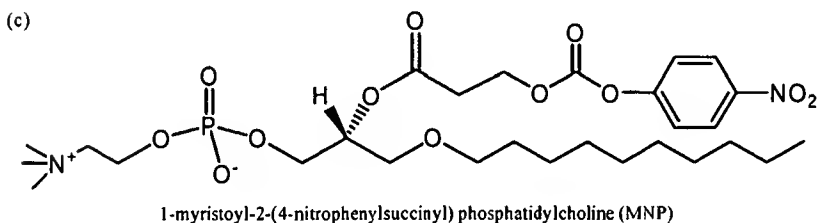
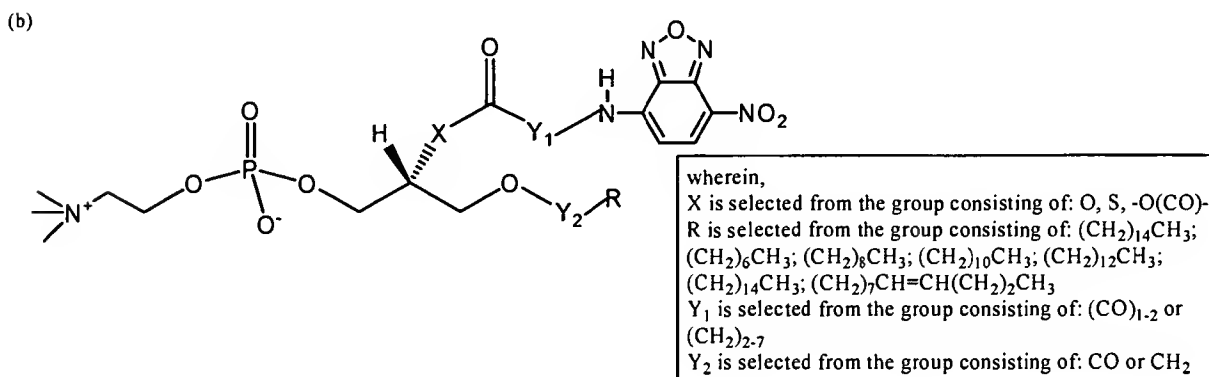
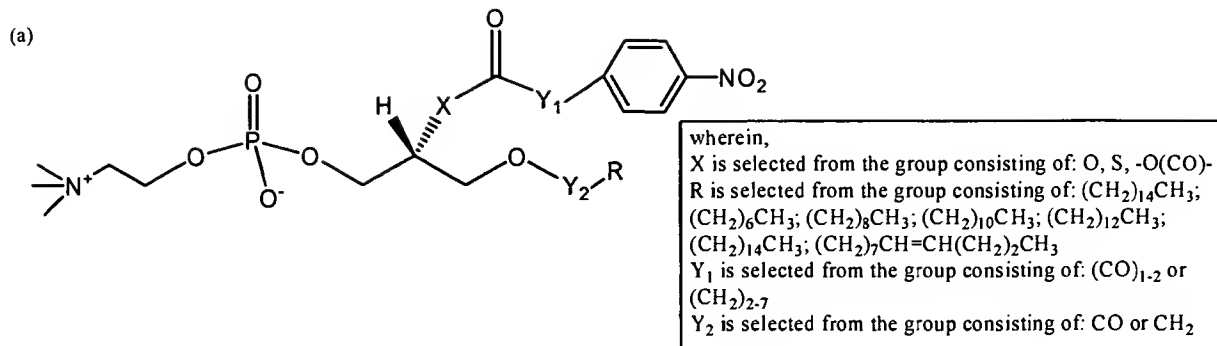
9. The method of claim 8 where in the substrate is an oxidized derivative of (a), (b), (c), (d) or (e).
10. The method of claim 1, wherein the detectable product has a radioactive, colorimetric, paramagnetic or fluorescent label.
- 5 11. The method of claim 1 wherein the detectable product is measured fluorimetrically, colorimetrically, paramagnetically or via radiation.
12. The method of claim 1 which further comprising comparing the measured detectable product of step (d) to detectable product in a control comprising an enzymatically active Lp-PLA2 standard.
- 10 13. The method of claim 12 wherein the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein.
14. The method of claim 13 wherein the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.
- 15 15. The method of claim 1 wherein the immobilized binder is bound to a multi-well plate, a magnetic bead, or a latex bead.
16. A method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising:
  - 20 (a) contacting an binder, which specifically binds Lp-PLA2, with the sample to form a binder-Lp-PLA2 complex;
  - (b) immobilizing the binder-Lp-PLA2 complex;
  - (c) washing the immobilized binder-Lp-PLA2 complex to remove an enzymatically active unbound material or an interfering substance(s);
  - 25 (d) contacting the immobilized bound Lp-PLA2 with a substrate converted to a detectable product in the presence of Lp-PLA2; and
  - (e) measuring detectable product indicative of enzymatically active Lp-PLA2 in the sample.
17. The method of claim 16, wherein the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample.
- 30 18. The method of claim 16, wherein the binder is an antibody.
19. The method of claim 18, wherein the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody.

20. The method of claim 19, wherein the monoclonal antibody is 2C10, 4B4, B200, B501, 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.
21. The method of claim 16 wherein the binder-Lp-PLA2 complex is immobilized by binding to an immobilized compound.
- 5 22. The method of claim 21 wherein the immobilized compound is an antibody, protein or compound capable of binding the binder-Lp-PLA2 complex.
23. The method of claim 22, wherein the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody.
- 10 24. The method of claim 23, wherein the monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody.
25. The method of claim 21 wherein the immobilized compound is bound to a multi-well plate, a magnetic bead, or a latex bead.
26. The method of claim 16 wherein the binder is conjugated to an immobilizing agent.
27. The method of claim 26, wherein the binder conjugated to an immobilizing agent is an antibody.
- 15 28. The method of claim 27, wherein the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody.
29. The method of claim 28, wherein the monoclonal antibody is 2C10, 4B4, B200, B501, 90D1E, 90E3A, 90E6C, 90G11D, or 90F2D.
- 20 30. The method of claim 26 wherein the immobilizing agent is an antibody, protein or compound capable of binding an immobilized compound.
31. The method of claim 30, wherein the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody.
32. The method of claim 31, wherein the monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody.
- 25 33. The method of claim 26 wherein the immobilizing agent is biotin.
34. The method of claim 26 wherein the immobilizing agent, conjugated to the binder-Lp-PLA2 complex, binds to an immobilized compound.
35. The method of claim 34 wherein the immobilized compound is bound to a multi-well plate, a magnetic bead, or a latex bead.
- 30 36. The method of claim 35 wherein the bound compound is an antibody, protein or compound capable of binding the conjugated immobilizing agent.

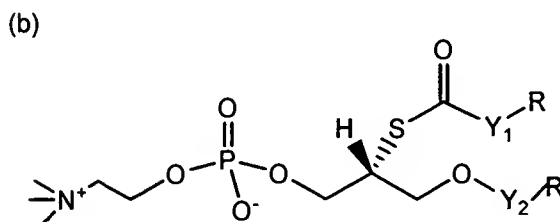
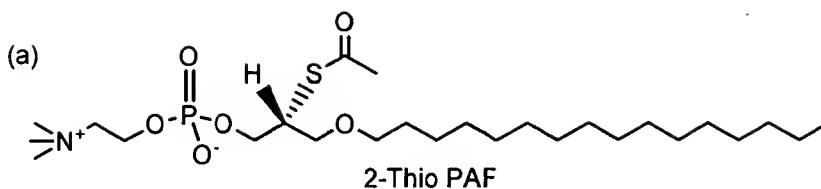
37. The method of claim 36, wherein the antibody is a monoclonal antibody, a phage display antibody, or a polyclonal antibody.
38. The method of claim 37, wherein the monoclonal antibody, the phage display antibody, or the polyclonal antibody is a rat, mouse or goat anti-Ig antibody.
- 5 39. The method of claim 36 wherein the bound substance is streptavidin.
40. The method of claim 16 wherein the enzymatically active unbound material is a phospholipase.
41. The method of claim 16 wherein the interfering substance(s) is a free-thiol compound.
- 10 42. The method of claim 16, wherein the substrate is selected from the group consisting of:



43. The method of claim 42 where in the substrate is an oxidized derivative of (a), (b), (c), (d) or (e).
44. The method of claim 16, wherein the detectable product has a radioactive, colorimetric, paramagnetic or fluorescent label.
- 5 45. The method of claim 16 wherein the detectable product is measured fluorimetrically, colorimetrically, paramagnetically or via radiation.
46. The method of claim 16 further comprising comparing the measured detectable product of step (e) to detectable product in a control comprising an enzymatically active Lp-PLA2 standard.
- 10 47. The method of claim 46 wherein the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein.
48. The method of claim 47 wherein the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.
49. A kit for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising a binder which specifically binds Lp-PLA2 and a substrate converted to a detectable product in the presence of Lp-PLA2.
- 15 50. The kit of claim 49 wherein the substrate is selected from the group consisting of:



51. The kit of claim 50 wherein the substrate is an oxidized derivative of (a), (b), (c), (d) or (e).
52. The kit of claim 49 further comprising an enzymatically active Lp-PLA2 standard.
53. The kit of claim 52 wherein the enzymatically active Lp-PLA2 standard is a  
5 recombinant Lp-PLA2 protein or a native Lp-PLA2 protein.
54. The kit of claim 53 wherein the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.
55. A method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising:  
10 (a) incubating the sample with a compound which reduces active thiol(s) in the sample;  
(b) contacting the incubated sample with a substrate converted to a free thiol product in the presence of enzymatically active Lp-PLA2; and  
(c) measuring free thiol product indicative of enzymatically active Lp-PLA2 in the  
15 sample.
56. The method of claim 55, wherein the sample is a serum sample, a plasma sample, an EDTA treated plasma sample or an EDTA treated serum sample.
57. The method of claim 55 wherein the compound which reduces active thiol(s) in the sample is DTNB.
- 20 58. The method of claim 55 wherein the sample is incubated at room temperature.
59. The method of claim 55 wherein the sample is incubated at 37°C.
60. The method of claim 55 wherein the sample is incubated from about 2 to about 120 minutes.
61. The method of claim 55 wherein the sample is incubated from about 5 to about 30  
25 minutes.
62. The method of claim 55 wherein the substrate is selected from the group consisting of:



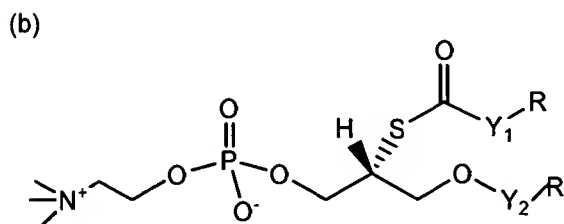
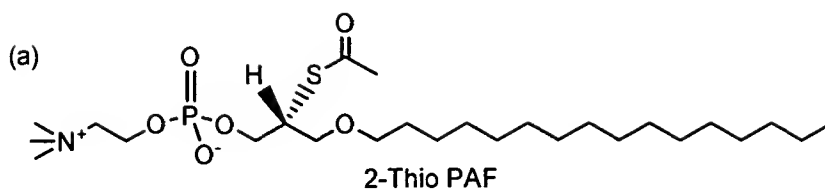
wherein,

R is selected from the group consisting of: (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>; (CH<sub>2</sub>)<sub>7</sub>CH=CH(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>

Y<sub>1</sub> is selected from the group consisting of: (CO)<sub>1-2</sub> or (CH<sub>2</sub>)<sub>2-7</sub>

Y<sub>2</sub> is selected from the group consisting of: CO or CH<sub>2</sub>

63. The method of claim 62 where in the substrate is an oxidized derivative of (a) or (b).
64. The method of claim 55 further comprising comparing measured free thiol product of step (c) to free thiol product in a control comprising an enzymatically active Lp-PLA2 standard.
65. The method of claim 64 wherein the enzymatically active Lp-PLA2 standard is a recombinant Lp-PLA2 protein or a native Lp-PLA2 protein.
66. The method of claim 65 wherein the recombinant Lp-PLA2 protein is expressed in a baculovirus expression system or a mammalian expression system.
67. The method of claim 55 wherein the steps (a), (b), and (c) are conducted in a multi-well plate.
68. A kit for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample comprising a compound which reduces active thiol(s) and a substrate converted to a detectable product in the presence of Lp-PLA2.
69. The kit of claim 68 wherein the substrate is selected from the group consisting of:



wherein,

R is selected from the group consisting of:  $(\text{CH}_2)_{14}\text{CH}_3$ ;  
 $(\text{CH}_2)_6\text{CH}_3$ ;  $(\text{CH}_2)_8\text{CH}_3$ ;  $(\text{CH}_2)_{10}\text{CH}_3$ ;  $(\text{CH}_2)_{12}\text{CH}_3$ ;

$(\text{CH}_2)_{14}\text{CH}_3$ ;  $(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_2\text{CH}_3$

$\text{Y}_1$  is selected from the group consisting of:  $(\text{CO})_{1-2}$  or  
 $(\text{CH}_2)_{2-7}$

$\text{Y}_2$  is selected from the group consisting of: CO or  $\text{CH}_2$

70. The kit of claim 69 where in the substrate is an oxidized derivative of (a) or (b).
71. The kit of claim 68 further comprising an enzymatically active Lp-PLA2 standard.
72. The kit of claim 71 wherein the enzymatically active Lp-PLA2 standard is a  
 5 recombinant Lp-PLA2 protein or a native Lp-PLA2 protein.
73. The kit of claim 72 wherein the recombinant Lp-PLA2 protein is expressed in a  
 baculovirus expression system or a mammalian expression system.

## ABSTRACT

This invention relates to a method for measuring enzymatically active Lipoprotein Phospholipase A2 (Lp-PLA2) in a sample. Further, this invention relates to a Hybrid Immunocapture method for measuring enzymatically active Lp-PLA2 in a sample.

- 5 Specifically, this invention relates to a Hybrid Immunocapture method for measuring enzymatically active Lp-PLA2 in a sample utilizing an enzymatically active Lp-PLA2 standard. In addition, this invention relates to a kit for measuring enzymatically active Lp-PLA2 in a sample. Specifically, this invention relates to a kit for measuring enzymatically active Lp-PLA2 in a sample containing an enzymatically active Lp-PLA2 standard.

# FIG. 1A and FIG. 1B Hybrid ImmunoCapture (HIC) Assay

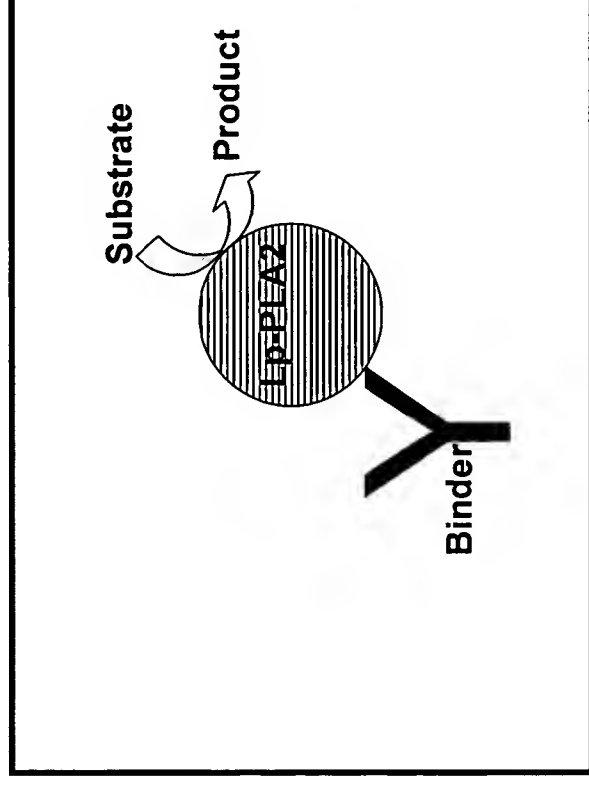


Fig. 1A

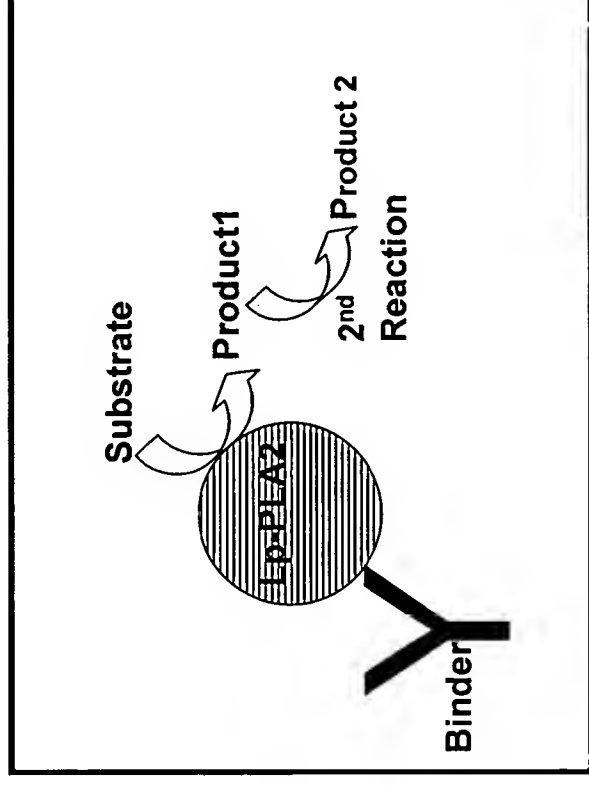
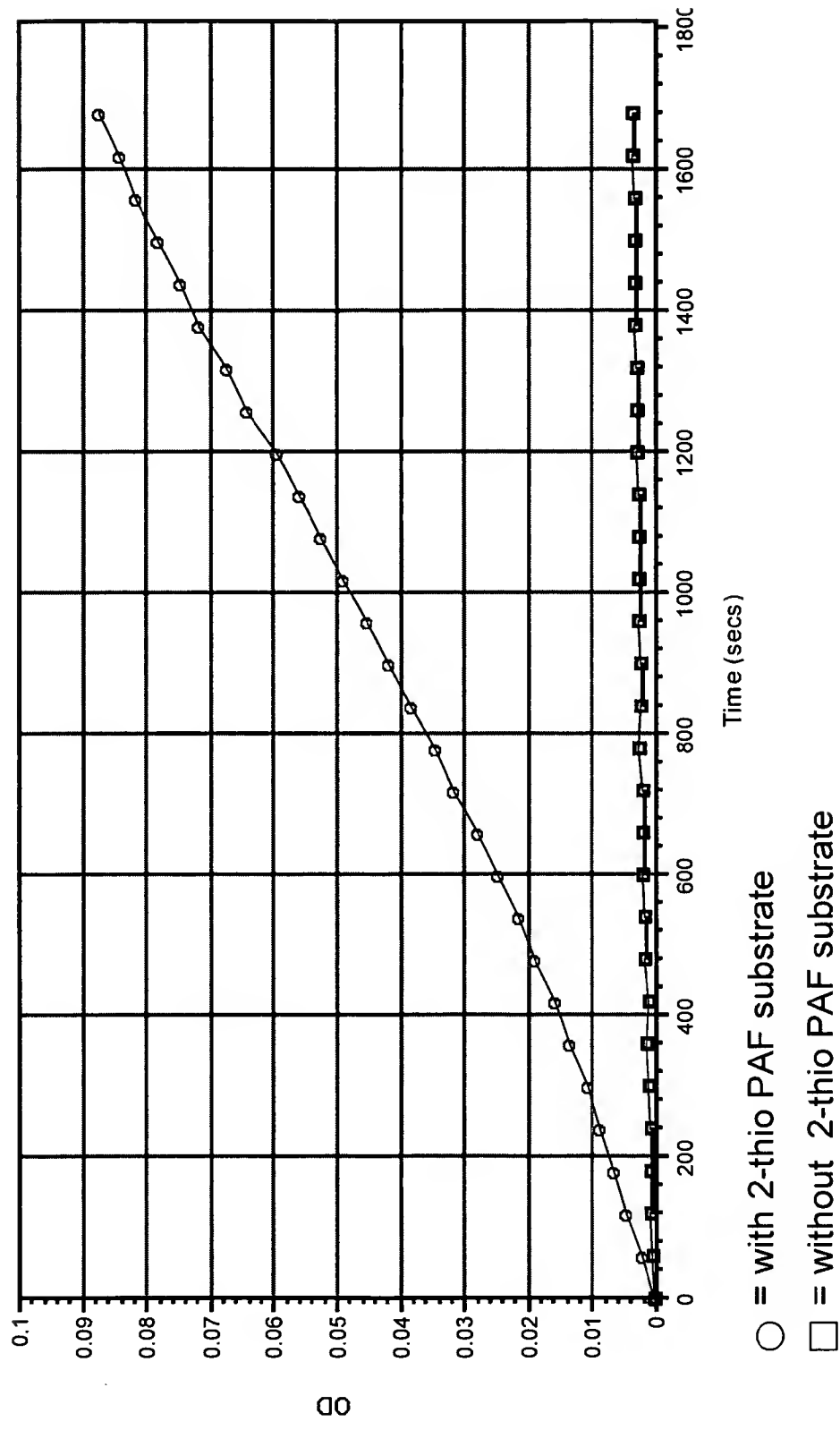
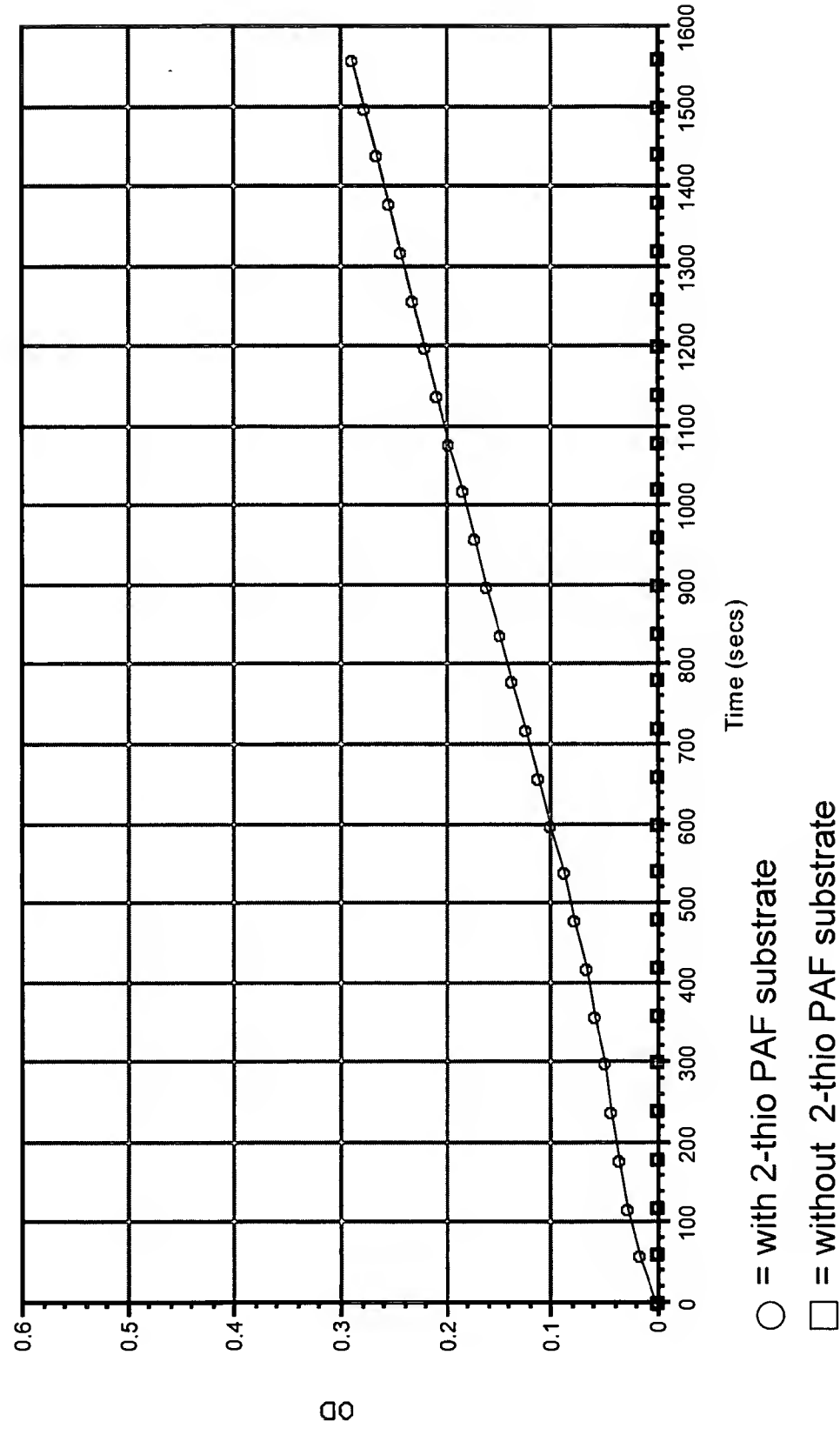


Fig. 1B

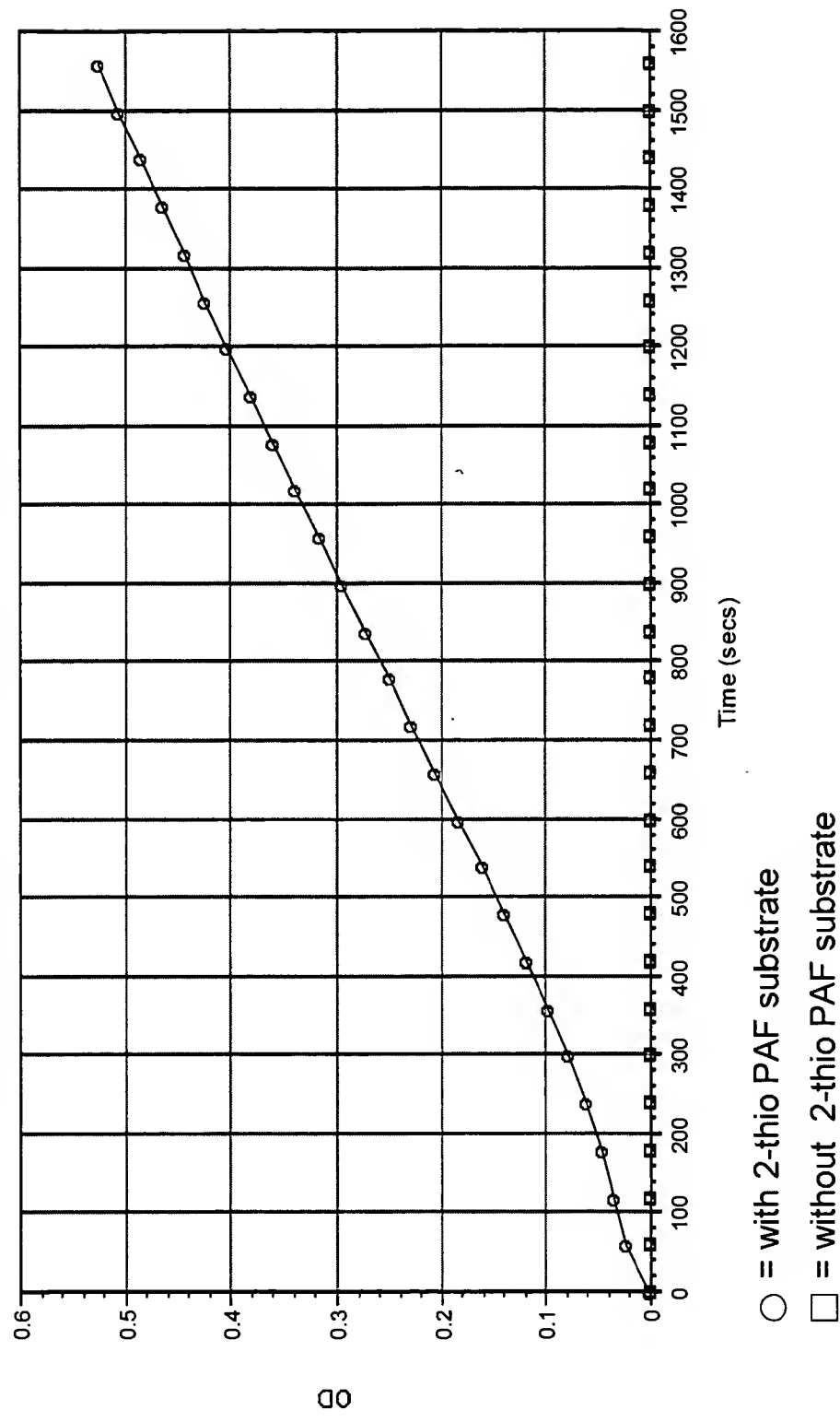
**FIG. 2: Plasma Lp-PLA2 Activity in HIC-ThioPAF Assay (2c10 as capturing mAb)**



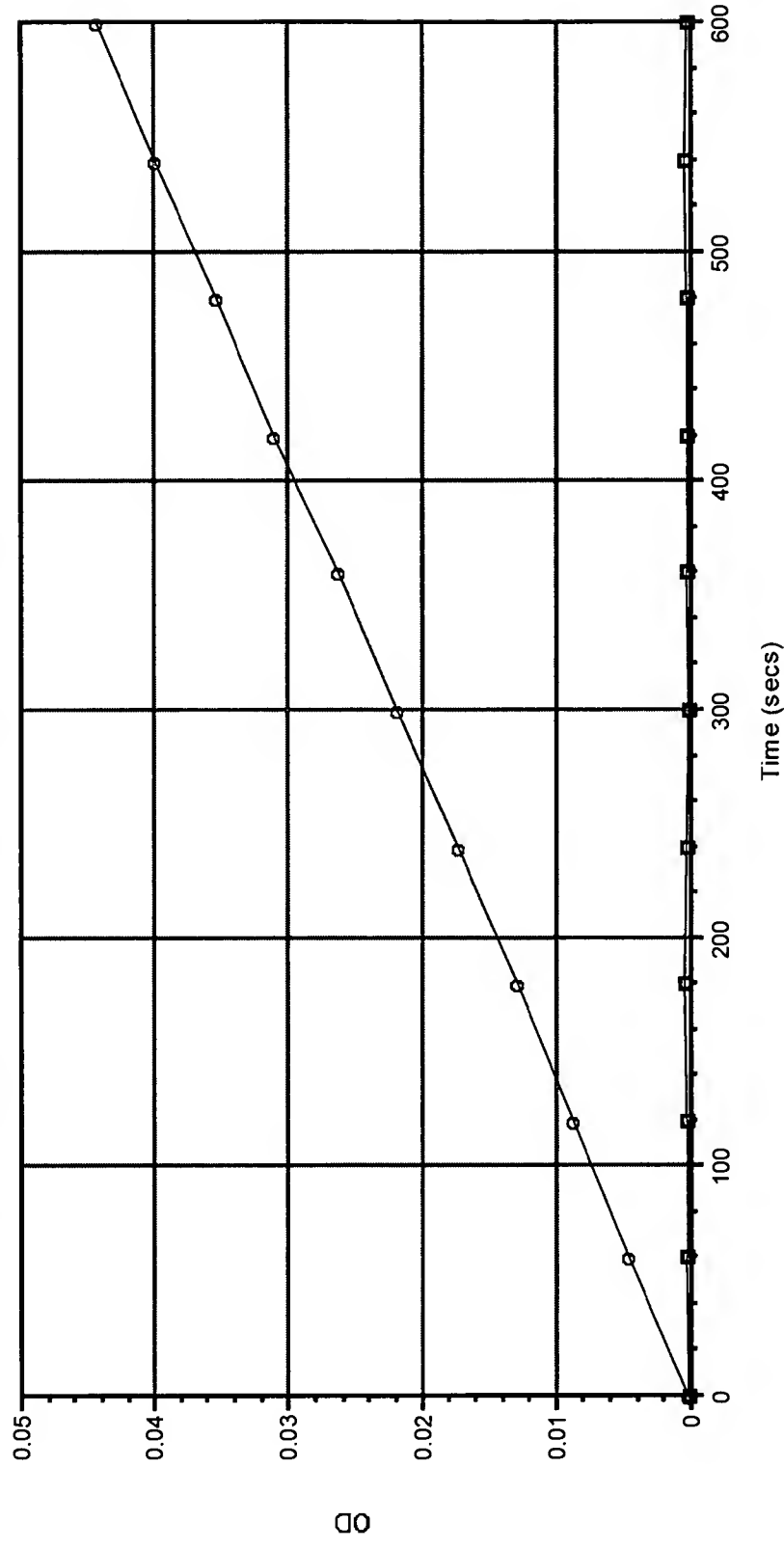
**FIG. 3: Plasma Lp-PLA2 Activity in HIC-ThioPAF Assay (B200.1 as capturing mAb)**



**FIG. 4: Plasma Lp-PLA2 Activity in HIC-ThioPAF Assay (B501.1 as capturing mAb)**

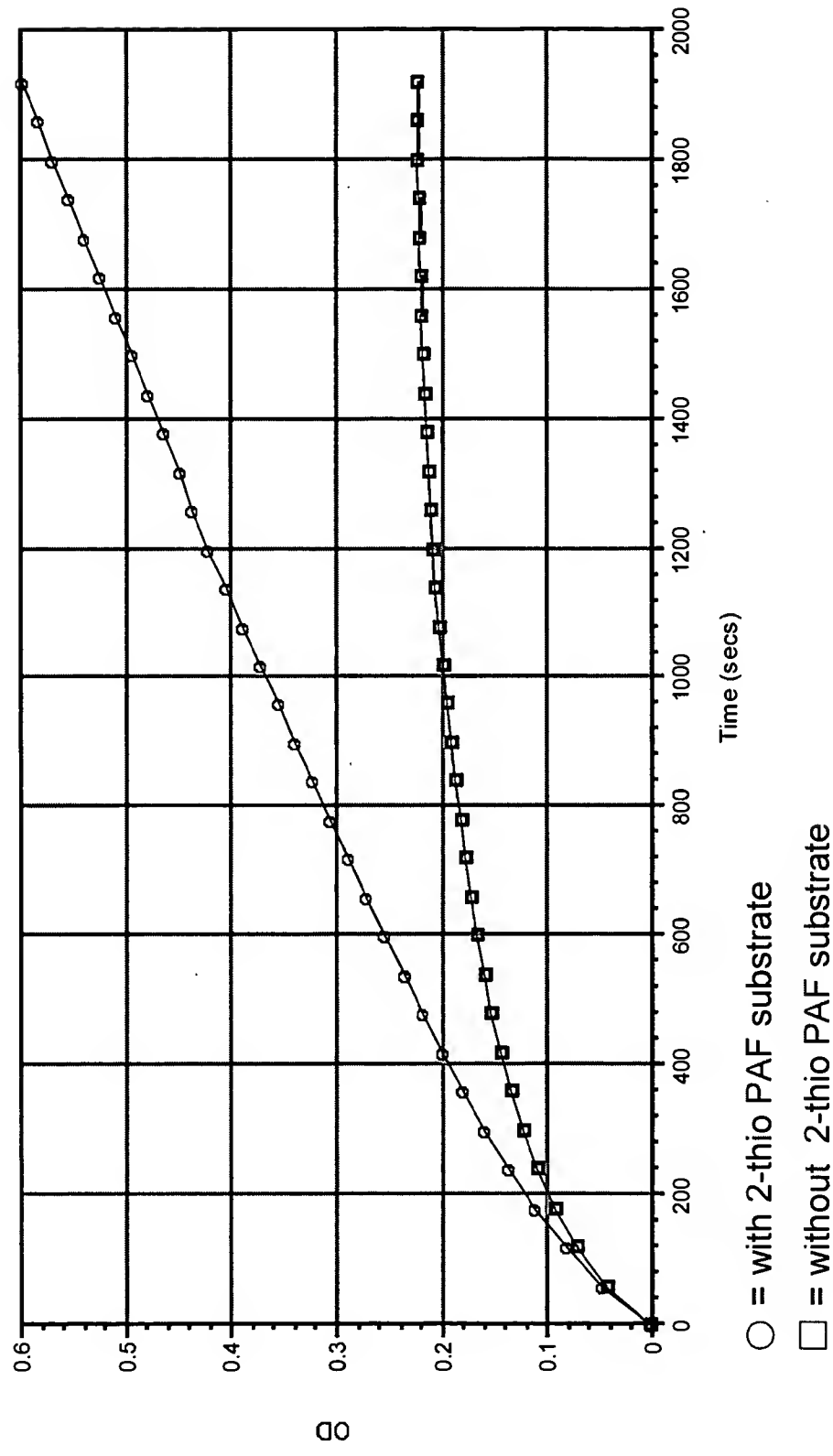


**FIG. 5: Plasma Lp-PLA2 Activity in HIC-MNP Assay (2c10 as capturing mAb)**

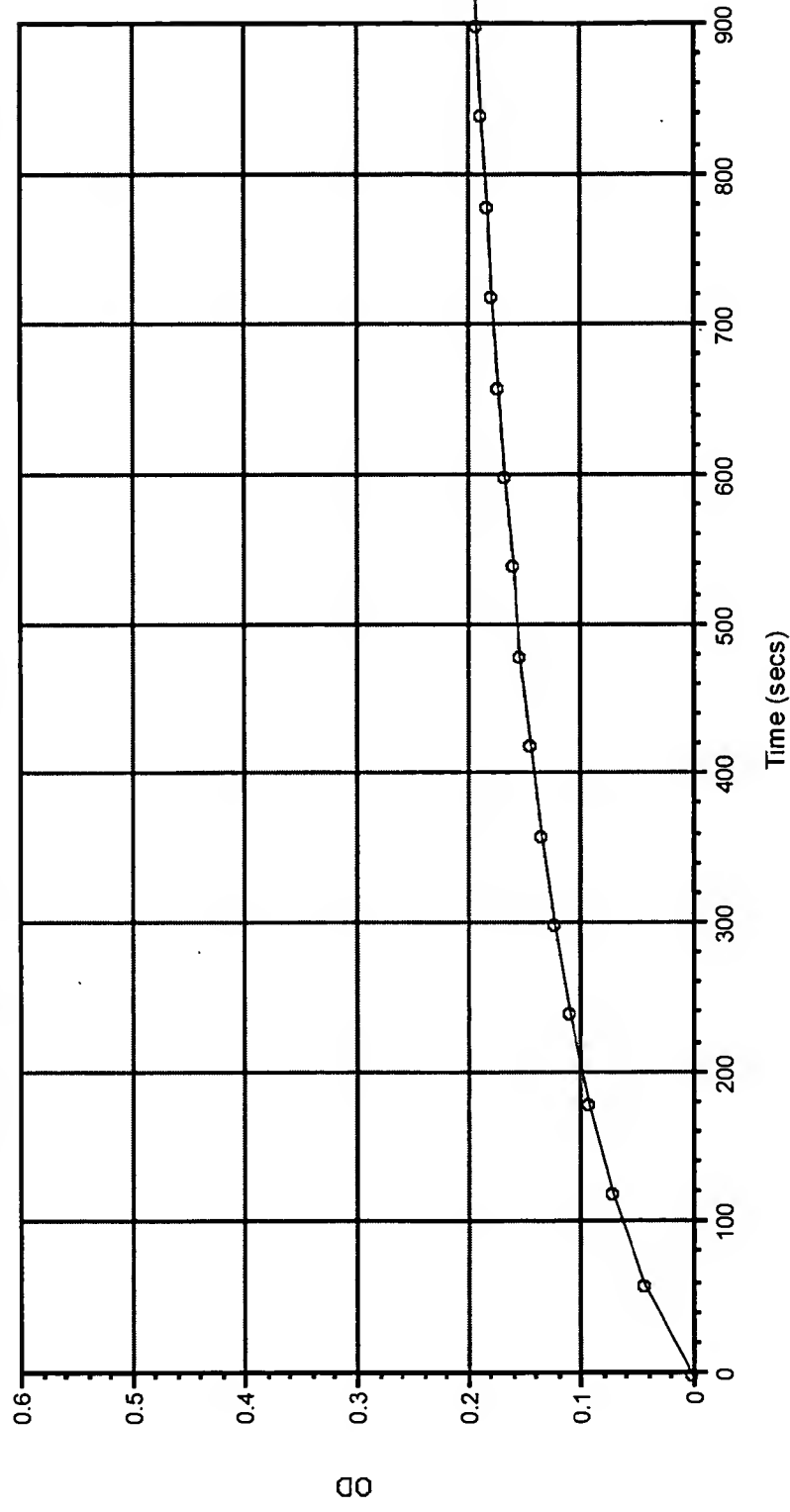


○ = with 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine substrate (MNP)  
 □ = without 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine substrate

**FIG. 6: Plasma Lp-PLA2 Activity In Commercial ThioPAF Assay**



**FIG. 7: Plasma sample background in Improved ThioPAF Assay, with DTNB but w/o substrate added**



○ = without 2-thio PAF substrate; with DTNB added

**FIG. 8: Plasma Lp-PLA2 Activity in Improved ThioPAF Assay**

